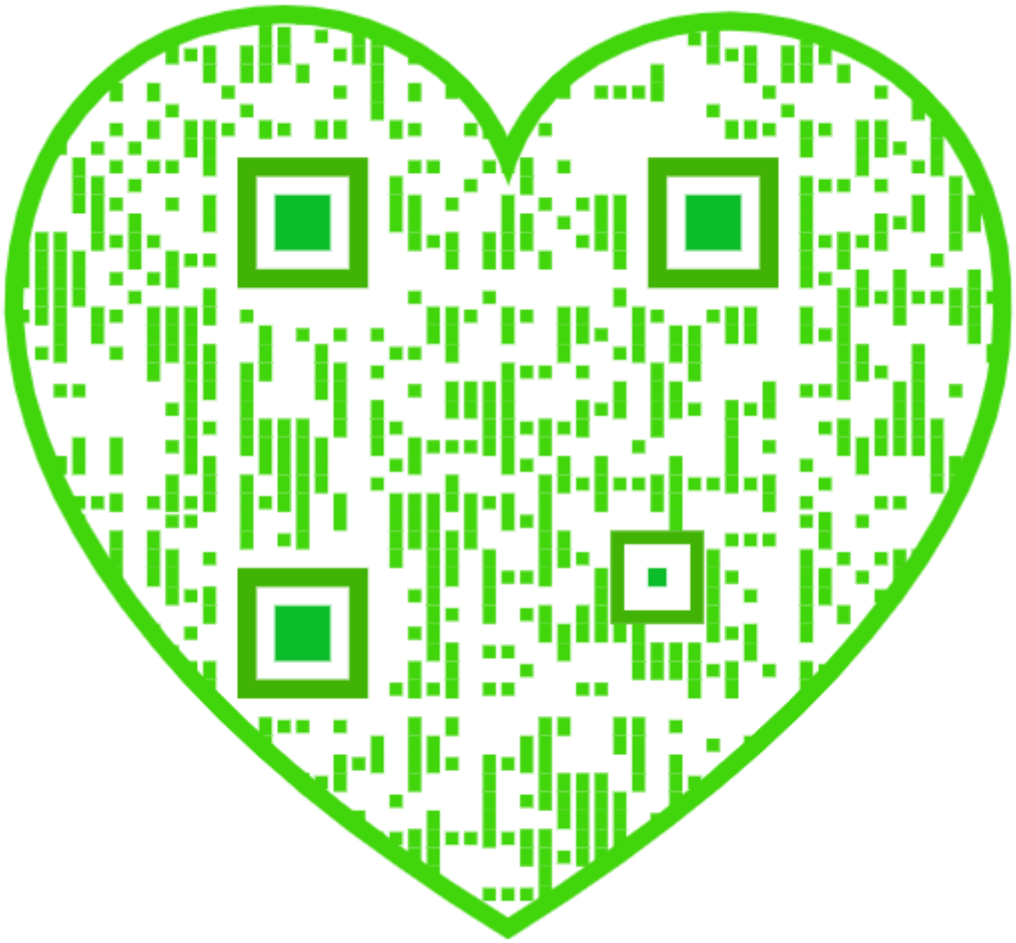

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A PRACTICAL MANUAL OF

MEDICAL AND BIOLOGICAL STAINING TECHNIQUES

The author in his laboratory.

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A PRACTICAL MANUAL OF MEDICAL AND BIOLOGICAL STAINING TECHNIQUES

by

EDWARD GURR

F.R.I.C., F.R.M.S., F.L.S., M.I.Biol.

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PREFACE TO FIRST EDITION

THIS book has been written because it is felt that there is an urgent need of a practical manual dealing with all or most branches of microscopic staining, entirely divorced from theory and general statements. It has been the writer's experience that a great deal of time is lost in searching through volumes of theory and general statements in order to extract a particular staining method which, even when found, may not be complete.

It is hoped that this book will form a useful supplement to the standard works on anatomy, bacteriology, biology, botany, cytology, embryology, entomology, histology, mycology, pathology, veterinary science, zoology, etc.

It should be pointed out that this book contains all the subject matter, revised, rearranged and co-ordinated, of the writer's own publications *Microscopic Staining Techniques* which were put out in the form of three booklets, No. 1, 2 and 3 as a temporary measure in an endeavour to meet the more pressing demands for information on the application of microscopic stains. The booklets attained a world-wide circulation and were in fact chosen by the British Council for inclusion in their exhibits of British medical books. The writer has since received many requests to incorporate all three parts of *Microscopic Staining Techniques* into one and this has been done in the present book, but with many additions.

Many of the methods given here are standard, some are not so well known, while others are unknown.

In writing this book, references have been made to numerous journals and standard works, chief of which were as follows:

Stain Technology (Biotech Publication, Geneva, N.Y., U.S.A.).
Plant Microtechnique, by D. A. JOHANSEN (McGraw-Hill, New York).
Handbook of Practical Bacteriology, by T. J. MACKIE and J. E. MCCARTNEY (E. & S. Livingstone, Edinburgh).
McClung's Handbook of Microscopical Technique, edited by RUTH MCCLUNG JONES (Paul B. Hoebber, Inc., New York, 16, U.S.A.).

Laboratory Technique, by E. V. Cowdry (Williams & Wilkinson, Baltimore, U.S.A.).

Histopathologic Technic, by R. D. Lillie (Blakiston Co., Philadelphia, U.S.A.).

Schaffer's Essentials of Histology, by H. M. Carleton and E. H. Leach (Longmans, Green & Co., London).

Pathological Technique, by F. B. Mallory (Saunders, Philadelphia, U.S.A.).

Histological Technique, by H. M. Carleton and E. H. Leach (Oxford University Press, England).

The section on Fluorescence Microscopy is a modification of the writer's paper which was published in *The Journal of the Royal Naval Medical Service*, 1951, Vol. xxxvii, No. 3, and thanks are due to the editor of that journal for permission to include the modified paper in this book.

I should like to place on record my thanks to my wife, F. P. Gurr, B.Sc., for her helpful criticism of the manuscript.

August, 1952

PREFACE TO THE SECOND EDITION

I am very happy that the first edition of this book has met with so kindly a reception; and I hope that this enlarged and revised edition will be of greater service to medical research workers and biologists all over the world.

Many additions have been made to the subject-matter of the book, and I wish to thank readers of the first edition for their helpful suggestions, most of which have been incorporated in this second edition.

A section of histochemical methods has been added as it is felt that this may be of service to various laboratory workers, including those engaged in cancer research, who may be interested in the rapidly expanding science of histochemistry, which offers a rich field for investigation.

The subject-matter of the book, as before, has been divided into various headings: some of the methods which might have been classified as " Cytological " have been placed under other headings because the particular methods are more frequently required by workers other than cytologists. For similar reasons, methods which might have been transferred to the histochemical section have been left under the same headings where they appeared in the first edition.

In addition to the standard works mentioned in the preface to the first edition, reference has also been made to *The Microscopist's Vade-Mecum* edited by J. Brontë Gatenby and H. W. Beams (J. & A. Churchill, Ltd., London), and *Microscopic Histochemistry* by George Gomori (University of Chicago Press, U.S.A.).

It was stated in a review in a Balkan journal, and correctly so, that the first edition of this book gave no information on the techniques used in eastern and south-eastern European countries. The reason for this is the scarcity here of the medical and biological literature of those countries. I am not a linguist, but I have painfully translated some hundreds of pages of French and German literature to find some missing link for inclusion in this present edition and, if any medical or biological laboratory workers

in any country in the world have any new or improved techniques that would prove to be of help to their fellow laboratory workers in other countries, I should be very pleased to be given the opportunity of considering such techniques for inclusion in the third edition of this work: I am most anxious that it should be of the greatest possible service to medical and laboratory workers throughout the world and information in any language concerning particular techniques will be welcomed.

It has been stated in a Netherlands journal in a review, of the first edition of this book, that Gram's Iodine and Lugol's Iodine are identical; I must correct this misleading statement which was evidently made in error: Gram's Iodine is not identical with Lugol's Iodine; the two formulae are given in this book.

I should like to express my appreciation of the very energetic co-operation afforded me by my publishers, and the efficient manner in which their production manager, Mr. R. G. Thixton, and the printers Messrs. J. W. Arrowsmith Ltd., have handled the complicated business of getting the second edition into print.

42, Upper Richmond Road West
East Sheen
London.

Edward Gurr

April, 1955

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SECTION 1—GENERAL METHODS

FIXATION

The main objects of fixation are:

(*a*) To kill the cells suddenly and uniformly so that they retain, as near as possible, the same appearance which they possessed in life.

(*b*) To preserve the tissues, cells, etc., by the inhibition of putrifactive and autolytic changes.

(*c*) To set and hold intra-cellular bodies, cells, etc., by precipitation in the positions which they occupied in life, thereby facilitating the closest possible study of the histology and cytology of the cells.

(*d*) To facilitate differentiation in the refractive indices of certain cell elements which would otherwise be invisible owing to the exceedingly narrow margin between the refractive index of one type of cell and that of other types.

(*e*) To render the cells and tissue constituents resistant to the subsequent processes such as dehydration, clearing, embedding, staining, etc., prior to their examination under the microscope.

(*f*) To facilitate proper staining of tissues. Here it should be mentioned that some fixatives act as mordants while others act as inhibitors for certain stains, and it is, therefore, of considerable importance that a suitable fixative should be employed for a particular staining technique or a particular staining technique should be chosen to suit material which has already been treated with a particular fixative. Recommendations as to suitable fixatives are given for most, if not all, of the staining techniques described in this book.

It is essential, if good results are to be achieved, that tissues should be removed from the body with the least possible delay and fixed immediately. Bacteria, protozoa and other unicellular organisms should be living at the instant of fixation. The following points must be observed in order to avoid failure, waste of time, effort and materials:

1. Pieces of tissue should, whenever possible, be cut into slices 2 to 6 mm. in thickness to permit penetration of the fixative throughout within a reasonable time.

2. The container in which the material is to be fixed should be of sufficient size to take the pieces of tissue without their folding or bending.

3. If large organs are to be fixed, large incisions should be made to allow thorough penetration of the fixing fluid.

4. A volume of the fixing fluid roughly about twenty to fifty times as great as that of the material to be fixed is necessary.

5. Material must not be left in the fixing fluid beyond the necessary time, but should be taken out, washed, dehydrated, cleared and embedded or stored in a fluid, suitable for the particular material, until it is required for embedding.

6. After fixation, and before proceeding to dehydration, careful washing out of the excess fixative is necessary, except in the case of alcohol which requires no washing out. In most cases running water is employed for this purpose, but for some tissue cells and cell constituents, and for some fixatives, alcohol must be used. In all cases, however, it is necessary to use liberal quantities of the liquids for washing out.

7. A fixative suitable to the material to be examined and compatible with the stains to be employed should be chosen, as disregard of this factor will, as previously stated, lead to failure and disappointment as well as waste of time, effort and materials: the importance of this rule cannot be over-emphasized.

(a) FIXATIVES

The number of these from which to choose is legion, although the number in everyday use is comparatively small. Details of some of the more commonly used of these are given below: formulae of others are given in the appendix.

Acetic Acid, Glacial

Recommended for:

Rapid fixation of strongly contracting organisms.

Technique:

Fix in the warm acid for a maximum period of fifteen minutes; then remove excess acid by washing in 30–50% alcohol.

Remarks:

Acetic acid glacial is rarely used alone: it causes the swelling of cell constituents, etc., and it is of most value when mixed with such substances as formalin, alcohol, mercuric chloride, etc., to counteract their shrinking effect.

Acetone

Recommended for:

Rapid fixation of brain tissue for rabies diagnosis. (R. D. Lillie, *Histopathologic Technique.*) It is also employed for fixing tissue enzymes, particularly phosphatases and lipases.

Technique:

Thin slices of tissue are fixed in pure acetone for twelve to twenty-four hours at 0° C.; they are then dehydrated by immersion in two changes of pure acetone for two hours in each at room temperature, and afterwards cleared by immersing for half an hour in each of two changes of benzene before infiltration with paraffin wax.*

Alcohol Absolute

Recommended for:

Glycogen, Amyloid, Fibrin, Hyaline, Haemofuscin, Phosphatase.

Technique:

Fix from two hours to several days according to the nature of the material; dehydrate; clear.

Remarks:

Unsuitable for fats and lipines as these are dissolved by the higher concentrations of alcohol.

* Gomori, *Proc. Soc. Exp. Biol. and Med.*, 58, 362, 1945.

Allen's Fixative (B.15)

Recommended for:

Chromosomes; plant tissues, particularly buds.

Formula:

Picric acid, saturated, aqueous	75 ml.
Formalin (formaldehyde 40%)	20 ml.
Acetic acid, glacial	5 ml.
Chromic acid	1·5 gm.
Urea	2 gm.

Technique:

Fix tissues from four to sixteen hours then immerse in frequent changes of 70% alcohol over a period of forty-eight hours or until the yellow coloration due to picric acid ceases to come out.

Remarks:

The solution deteriorates very rapidly and it is, therefore, essential that this fixative should be prepared only when it is required for immediate use.

Bouin's Fluid

Recommended for:

Embryological specimens, for elementary bodies, Purkinje cells, Argentaffin reaction, and for animal tissues in general.

Formula:

Picric acid, saturated aqueous	75 ml.
Formalin	25 ml.
Glacial acetic acid	5 ml.

Technique:

Fix from eighteen hours to two days; then wash in 50% followed by 70% alcohol until most of the yellow coloration, due to picric acid, is extracted. Alternatively the picric acid can be washed out with the alcohols after the tissues have been embedded and sectioned.

It is not essential that all the picric acid, which serves as a

mordant enhancing many staining effects, should be entirely extracted from the fixed tissues.

Remarks:

This fixative, which keeps indefinitely and causes only slight shrinkage of tissues, is compatible with almost every staining technique: it is considered to be a valuable fixative for most purposes, although it is unsuitable for kidney and mucin. Its penetration power is great, and delicate material should be left in contact with this fixative only for the minimum time, to avoid over-fixation: this applies to cytological work in particular. There are many modifications of Bouin, of which Allen's Fixative, B.15, has proved to be the most satisfactory for chromosomes in mammalian tissues.

Bouin - Duboscq (Duboscq-Brasil, or Alcoholic Bouin)

Recommended for:

Arthropods containing parasites and protozoan cysts, and for chitinous tissue.

Formula:

Absolute alcohol	48 ml.
Formalin (formaldehyde 40%) ..	30 ml.
Glacial acetic acid	7·5 ml.
Picric acid, saturated aqueous ..	12·5 ml.
Distilled water	15 ml.

Technique:

Fix from eighteen hours to two days; then wash in 70% alcohol.

Remarks:

This is stated to be more penetrating than aqueous Bouin and for this reason it is employed for hard tissues.

Carnoy's Fluid

Recommended for:

Glycogen; for animal tissues in general, and for plant cytology.

Formula:

Absolute alcohol	60 ml.
Chloroform	30 ml.
Glacial acetic acid	10 ml.

Technique:

ANIMAL TISSUES. Fix for one and a half to two hours; then transfer to two changes of absolute alcohol before clearing and embedding.

PLANT TISSUES. Fix root tips for a quarter-of-an-hour; anthers, for one hour.

Remarks:

A rapid fixative with great penetrating power.

Carnoy - LeBrun Fluid

Recommended for:

Insects and ticks, and as a rapid and penetrating fixative for plant tissues.

Formula:

Absolute alcohol	30 ml.
Glacial acetic acid	30 ml.
Chloroform	30 ml.
Mercuric chloride ..	to saturation (about 10 gm.)

Technique:

Fix from half to one minute; then wash in 95% alcohol.

Remarks:

Not recommended for routine fixation of plant tissues.

Champy's Fluid

Recommended for:

Plant and animal tissues in general; mitochondria and other cytological detail.

Formula:

Potass. dichromate 3% aqueous ..	35 ml.
Chromic acid 1% aqueous	35 ml.
Osmic acid 2%	20 ml.

Technique:

Fix for six to twenty-four hours; then wash in running water for the same length of time.

Flemming's Fluid (Strong)

Recommended for:

Plant and animal tissues, for cytological detail; for cellular structures; and for fat.

Formula:

A.	Chromic acid 1%	30 ml.
	Acetic acid, glacial	2 ml.
B.	Osmic acid 2%	8 ml.

Mix A and B immediately before use.

Technique:

Fix for one to twenty-four hours according to the material; for chromosomes an hour is sufficient. Wash with running water for twenty-four hours.

Remarks:

The penetration power of this fixative is poor; and that of the weak solution (*see* below) is poorer still; however, either solution gives good results with basic stains, particularly safranin, and iron haematoxylin. Quite apart from their high cost, Flemming fixatives should definitely not be employed as general fixatives, but only in special cases for very small pieces of tissue where fixation extending through a layer of about four or five cells in thickness is sufficient, as this is the limit of their penetrating power even in loose-celled tissues.

Flemming's Solution (weak)

Recommended for:

All purposes for which Flemming's strong solution is used.

Formula:

A.	Chromic acid 1%	25 ml.
	Acetic acid 1%	10 ml.
B.	Osmic acid 1%	10 ml.
	Distilled water	50 ml.

Technique:

Used in the same way as the strong solution (*see* above), except that the volume of the fixative required is about eight or ten times that of the material to be fixed.

Remarks:

See under Flemming's Fluid (strong).

Formalin Neutral

Recommended for:

Animal tissues in general, particularly nervous tissue.

Formula:

Formalin (formaldehyde 40%) ..	100 ml.
Tap water	900 ml.
Magnesium carbonate	to excess

Shake well and allow to stand several hours at least; then decant off the volume of the clear fluid required for fixation.

Technique:

Fix at least twenty-four hours at room temperature, or six to eight hours at 50–60° C. Washing out is unnecessary.

Remarks:

Formalin penetrates well, tissues may be kept in it for long periods without undue hardening, although there is a gradual decrease in basophilia of cytoplasm and nuclei, and certain cytoplasmic structures are not hardened by it sufficiently to permit paraffin embedding. Best adapted to material which is to be embedded in celloidin rather than in paraffin wax; also suitable for frozen sections.

Formalin Buffered

Formula:

Neutral formalin, as above	1 litre
Sodium dihydrogen phosphate, monohydrate, A.R.	4 gm.
Disodium phosphate anhydrous, A.R.	6·5 gm.

Technique:

As for neutral formalin.

Remarks:

Neutral formalin turns acid on keeping owing to the production of formic acid, whereas the buffered fixative remains neutral.

Recommended for:

All purposes for which neutral formalin is employed where a neutral fixative is required.

Formol - Alcohol

Recommended for:

Glycogen in animal tissues. Fibrin. Peroxidase. Plant tissues, particularly pollen tubes in styles.

Formula:

Formalin (40% formaldehyde) ..	100 ml.
Alcohol 70%	900 ml.

Technique:

Fix for three to six hours; then dehydrate, clear and embed. Alternatively, if it is not convenient to dehydrate, clear and embed at once, the tissues may be stored for long periods without deleterious effects in 70% alcohol.

Remarks:

This fixative, which penetrates quickly, while compatible with most stains, is particularly suitable for indigo carmine.

Helly's Fluid

Recommended for:

Animal tissues in general, but particularly for blood-forming organs.

Formula:

Potassium dichromate	5 gm.
Mercuric chloride	10 gm.
Sodium sulphate crystals	2 gm.
Formalin (40% formaldehyde) ..	10 ml.
Distilled water	200 ml.

N.B.—The formalin should not be added until the fixative is required for immediate use.

Technique:

Fix for twelve to twenty-four hours. Wash in running water for the same time. Transfer to 80% alcohol; dehydrate; clear and embed.

Remarks:

Not recommended for bacteria, cytoplasm degeneration, necrosis or regeneration.

Hermann's Fluid

Recommended for:

Cytological work.

Formula:

Platinic chloride 10%	6 ml.
Osmic acid 1%	32 ml.
Glacial acetic acid	4 ml.
Distilled water	38 ml.

N.B.—The solution should be freshly prepared.

Technique:

Fix from twelve to sixteen hours, wash in running water for three to six hours; then treat as for Flemming fixed-material.

Remarks:

While this fixative mordants chromatin for staining with basic stains, it inhibits staining with acid stains. Good plasma staining is difficult if not imposssible after this fixative.

Kleinenberg's Fluid

Recommended for:

Embryos; marine organisms, arthropods, chitinous material.

Formula:

Sulphuric acid 1%	100 ml.
Picric acid, saturated aqueous ..	49 ml.

Technique:

Wash out the picric acid with warm 70% alcohol, followed by increasing strengths of alcohol.

Remarks:

This fixative is a powerful penetrant of chitin.

Lewitsky's Fluid

Recommended for:

Plant cytology.

Formula:

Formalin (formaldehyde 40%) ..	100 ml.
Distilled water	100 ml.
Chromic acid 5% aqueous	100 ml.

Technique:

Fix tissue for twelve to twenty-four hours; then wash for six to sixteen hours in running water.

Marchi's Fluid

Recommended for:

Animal and plant tissues generally.

Formula:

Potassium dichromate 2·5% aqueous	100 ml.
Sodium sulphate crystals	1 gm.
Osmic acid 1% aqueous	50 ml.

Note.—The last item should be added immediately before use.

Technique:

Immerse thin pieces of tissues, not more than 2 mm. thick in the fixative for four to eight days; then wash in running water for twelve to sixteen hours. Transfer to 70% alcohol, afterwards dehydrating, clearing and embedding in the usual way.

Remarks:

This fixative is also employed to blacken nerve fibres.

Navashin's Fluid

Recommended for:

Cytological study of plant tissues.

Formula:

Chromic acid	1·5 gm.
Acetic acid 10% aqueous	100 ml.
Distilled water	60 ml.
Formalin (formaldehyde 40%) ..	40 ml.

Note.—The formaldehyde should not be added until the solution is required for immediate use.

Technique:

Fix material for one to two days; then wash twelve to sixteen hours in running water. Dehydrate; clear and embed.

Orth's Fluid

Recommended for:

Demonstration of acute degenerative processes to be stained with Giemsa, Wright, or Leishman Stain, and for Intestine.

Formula:

Potassium dichromate	2·5 gm.
Sodium sulphate crystals	1 gm.
Distilled water	100 ml.
Formalin (formaldehyde 40%) ..	10 ml.

The last item should not be added until the fixative is required for immediate use.

Technique:

Fix pieces of tissue up to 1 cm. in thickness for two to four days; then wash in running water from twelve to twenty-four hours. Transfer to 80% alcohol; dehydrate; clear and embed.

Remarks:

This fixative may be employed as a general fixative for animal tissues. It is useful where a firm consistency of tissue is required, but it is not recommended for sharp histological detail. The value of the sodium sulphate in the above formula is extremely doubtful and it appears that this constituent may be left out without any noticeable effect.

Petrunkevitch's Cupric Paranitrophenol Fixative*

Recommended for:

Tissues in general.

Formula:

Alcohol 60%	100 ml.
Nitric acid, pure (sp. gr. 1·41—1·42)	3 ml.
Ether	6 ml.
Cupric nitrate $Cu(NO_3)$ 2, $3H_2O$ ..	2 gm.
Paranitrophenol pure, cryst... ..	5 gm.

Technique:

Fix material for twelve to twenty-four hours. Wash in 70% alcohol; dehydrate; clear and embed.

Remarks:

All stains commonly in use may be employed after this fixative, which causes less hardening than most other fixatives.

Regaud's Fluid

Recommended for:

Mitochondria and rickettsia in animal tissues.

Formula:

Potassium dichromate	3 gm.
Distilled water	100 ml.
Formalin (formaldehyde 40%) ..	25 ml.

Technique:

Fix material for three days changing the fluid every day. Immerse in 3% potassium dichromate for six to eight days; then wash in running water for twenty-four hours. Dehydrate; clear and embed.

Remarks:

Suitable for Giemsa stain and for Masson's trichrome stain.

Schaudinn's Fluid

Recommended for:

Animal tissues in general. Protozoa.

*A. Petrunkevitch, *Science* 77, 117-18, 1933.

Formula:

Mercuric chloride saturated aqueous	66 ml.
Absolute alcohol	33 ml.
Glacial acetic acid	1 ml.

N.B.—The acid should be added immediately before use.

Technique:

Fix tissues for six to sixteen hours. Wash in several changes of 70% alcohol.

Remarks:

The fixative may be used at about 65° C. when less time is required for its penetration.

Susa Fluid (Heidenhain)

Recommended for:

Animal tissues in general.

Formula:

Mercuric chloride, saturated aqueous	50 ml.
Trichloracetic acid	2 gm.
Formalin (formaldehyde 40%) ..	20 ml.
Distilled water	30 ml.
Glacial acetic acid	4 ml.

Technique:

Fix for five to twelve hours; then wash out with 95% alcohol.

Remarks:

Compatible with most stains, but not with Weigert's elastin stain. Susa offers the advantage over most other fixatives in that it causes less shrinkage and less hardening, thereby rendering tissues easier to cut.

Zenker's Fluid

Recommended for:

Perfect histological detail in animal tissues in general.

Formula:

Potassium dichromate 2·5% aqueous	100 ml.
Mercuric chloride	5 gm.
Glacial acetic acid	5 ml.

N.B.—The acid should not be added until the fixative is required for immediate use.

Technique:

Immerse slices of tissue in the fluid for six to twenty-four hours according to the nature of the material and the thickness of the slices. Wash in running water for twelve to twenty-four hours; then transfer to 80% alcohol.

Remarks:

Unsuitable for Mitochondria. Suitable for Mallory's connective tissue stain; for demonstration of Muscle, Fibrin, Haemofuscin, Purkinje cells, etc.

(b) DEHYDRATION, CLEARING, EMBEDDING, SECTIONING, ETC.

CELLOIDIN METHOD FOR EMBEDDING TISSUES

For preserving the relations of cell layers of different consistency, as are contained in the eye; for large objects; for pieces of central nervous system; and for hard tissues such as decalcified bone.

Solutions required:

A. *Celloidin* 8%

Celloidin flakes	25 gm. damped with absolute alcohol*
Absolute alcohol	150 ml.
Ether	163 ml.

**Note:* This may be obtained in 25-gm. bottles, ready damped with absolute alcohol.

Pour the alcohol and ether into a clean, absolutely dry, wide-mouth bottle of about 32 ounce capacity, and mix by inverting the bottle several times, releasing the stopper at intervals, to release the pressure of ether vapour; then add the Celloidin flakes and invert the bottle as before. Leave for about 12 hours to dissolve; inverting or shaking the bottle gently at intervals.

B. *Celloidin* 4%

Celloidin solution 8%	2 volumes
Absolute alcohol	1 volume
Ether	1 volume

Mix as described above, in a large-stoppered wide-mouth bottle.

C. *Celloidin* 2%

Celloidin 4%	2 volumes
Absolute alcohol	1 volume
Ether	1 volume

D.

Cedarwood oil, for clearing ..	1 volume
Chloroform	1 volume

Technique:

(*a*) **Wet Method**

1. Pieces of tissue not thicker than 5 mm. are fixed in the usual manner.

2. Wash in running water for the prescribed time for the particular fixative. If a fixative containing mercury has been used, remove mercurial precipitate by the standard technique.

3. Immerse the pieces of tissue for two hours in each of the following: 50%, 70% and 90% alcohol.

4. Immerse in absolute alcohol from two to sixteen hours, according to the nature and thickness of the tissue.

5. Immerse for twenty-four hours in a mixture consisting of equal volumes of absolute alcohol and ether, in a stoppered wide-mouth bottle, which must be absolutely dry.

6. Impregnate with 2% Celloidin solution from five to seven days.

7. Transfer to 4% Celloidin for five to seven days.

8. Impregnate with 8% Celloidin for three or four days.

9. The tissue is then taken out of the Celloidin and put into a mould made by folding a piece of writing paper, and the whole is then placed in a desiccator and left for several days, lifting the desiccator lid for a few seconds each day to accelerate the hardening of the Celloidin. If, through shrinkage of the Celloidin during this process, the tissue becomes exposed, pour on more Celloidin solution to cover it. Hardening of the block may be hastened by placing 1–2 ml. of chloroform in the bottom of the desiccator.

The block is hard enough for sectioning when no impression is left after pressing with the ball of the thumb.

10. The base of the hardened Celloidin block is dipped into 8% Celloidin then fixed to a roughened wooden or a vulcanite block by pressing firmly, afterwards leaving for at least half an hour with a weight on top.

11. Expose to chloroform vapour for half an hour; then attach the wooden or vulcanite block to the microtome holder, or store the Celloidin block mounted on the wooden or vulcanite block in 80% alcohol until required for sectioning.

12. The microtome knife and the Celloidin block must be kept moist with 70% alcohol and each section as it is cut must be transferred by means of a camel-hair brush, moistened with 70% alcohol, into a suitable vessel containing 70% alcohol in which the sections can be stored indefinitely until required for staining.

13. When required for staining the sections should be removed from the 70% alcohol by means of a small camel-hair brush, or a piece of thin glass rod bent at one end, and transferred to a series of watch glasses containing the reagents and stains, arranged on the bench in the order in which they are to be used. For instance, if it is desired to stain the sections with Haematoxylin and Eosin, the steps are as follows:

14. Immerse sections in 50% alcohol for a few minutes; then transfer to water.

15. Stain with Ehrlich Haematoxylin by the standard technique.

16. Blue in tap water; then stain in Eosin (aqueous solution).

17. Transfer to 70% alcohol; then immerse for five minutes in each of two lots of 96% alcohol.

Note: Absolute alcohol must be avoided as Celloidin is dissolved by it.

18. Immerse for five minutes each in two lots of Carbol-Xylol.

19. Pass into two changes of xylol.

20. Mount in balsam or Dammar-Xylol.

(*b*) Dry Method

1. Proceed exactly as described above up to and including step No. 8; then take the tissue out of the Celloidin and put it into a paper mould as described in step No. 9 (above).

2. Place the block in a desiccator for a day, lifting the lid for a few seconds every hour or so; then leave in the desiccator overnight.

3. Next morning place the block in a mixture consisting of equal volumes of cedarwood oil and chloroform and add another 8 volumes of cedarwood oil, one volume at a time, every hour for small objects, or every day in the case of large objects. The Celloidin should now be wholly transparent.

4. Fix the Celloidin block to the wooden or vulcanite block as described in step 10 (above).

5. Sections may now be cut without the necessity of moistening the knife or the block.

Note: Blocks prepared by this method are stored in a dry wide-mouth stoppered bottle.

CELLOIDIN - PARAFFIN WAX (Double Embedding)

For serial sections embedded in Celloidin

Solutions required:

Celloidin 1% in Methyl Benzoate

This is prepared by adding the appropriate weight of air-dried Celloidin flakes to a quantity of Methyl Benzoate in a clean dry

corked flask or bottle. Shake well; allow the flask or bottle to stand upright for an hour or so; then leave it inverted for an hour, afterwards leave it lying horizontally for a time; then stand it upright again, and repeat the process at intervals throughout the day and leave the bottle lying on its side overnight: next morning, solution should be complete and it is only necessary to shake the bottle well to ensure a homogeneous solution.

Technique:

1. Tissues are fixed and washed in running water, and any mercurial precipitate removed in the usual manner.

2. Immerse for two hours in each of the following: 50%, 70% and 90% alcohol.

3. Transfer to absolute alcohol for two to sixteen hours.

4. Immerse in Methyl Benzoate - Celloidin solution for twenty-four hours, at the end of which time pour off the solution and replace with a fresh lot in which the tissue should remain for a further forty-eight hours: if the tissue is not now clear, transfer it to a fresh lot of Methyl Benzoate - Celloidin solution for a further period of seventy-two hours.

5. Immerse in three changes of pure benzene, for four hours in the first lot, eight hours in the second, and twelve in the third.

6. Transfer to a mixture consisting of equal parts of paraffin wax and pure benzene in the embedding oven for an hour.

7. Immerse in two changes of pure paraffin wax from a quarter of an hour to six hours in each, depending upon the thickness and the nature of the tissue.

Note: It is of utmost importance that tissues should be kept in the embedding oven just long enough for the paraffin wax to penetrate fully. Prolonged heating in the oven causes shrinking and hardening of the tissues rendering sections difficult to cut. If, on the other hand, any of the Methyl Benzoate remains in the tissue and sufficient time has not been allowed for proper penetration of the paraffin wax satisfactory sections cannot be cut. It is best, whenever possible, to cut thin slices of tissue for embedding, preferably not more than 5 mm. thick so that the total time for impregnating in the pure paraffin wax need be no longer than three hours. Large objects such as whole embryos need a total

time of twelve hours in pure paraffin wax. Pieces of brain and spinal cord about 5 to 10 mm. thick, skin, and large objects such as whole embryos need at least three changes of pure paraffin wax for a total time of about 12 hours, whereas organs such as spleen, containing a large amount of blood, muscle, fibrous tissue, require no longer than a total of three hours in the paraffin baths. It is, however, only by experience that the technique of embedding can be mastered.

8. Cast the tissue in an embedding mould and proceed as in the case of paraffin sections (*see* page 32).

CELLOIDIN - PYRIDIN

A rapid method of dehydrating, clearing and embedding, which obviates the use of alcohols and the consequent hardening of tissues

Reagents required:

A. Pyridin, extra pure, redistilled .. 1 volume
Celloidin 4% in equal volumes of absolute alcohol and ether .. 1 volume

B. Celloidin 8% in equal volumes of absolute alcohol and ether.

Technique:

1. Pieces of tissue are fixed in the usual manner.

2. Wash in running water for the prescribed time for the particular fixative employed. If a fixative containing mercury has been used, remove mercurial precipitate by the standard technique.

3. Immerse the tissue in two changes of Pyridin, from two to eight hours in each, according to the nature and the thickness of the tissue.

4. Immerse for twenty-four hours each in two changes of a mixture consisting of equal volumes of Pyridin and 4% Celloidin (formula as above).

5. Immerse in 8% Celloidin for twelve hours.

6. The tissue is then removed from the Celloidin bath, blocked and cut into sections by the standard technique described on pages 19-20.

FROZEN SECTIONS

For the identification of fat in tissues; for certain impregnation methods for the central nervous system; and for the rapid examination of pathological material, such as pieces of tumour, which may be sectioned, stained and diagnosed within a few minutes of their removal by the surgeon in the operating theatre

Sections as thin as 5μ may be cut, and an advantage of this method is that there is a lesser degree of shrinkage than in the case of paraffin-embedded material. It is not, however, possible to cut serial sections by this method, and sections cannot be stored before staining as in the case of paraffin-embedded material. Frozen sections should be employed only for the specific purposes mentioned above and not as an alternative to paraffin embedding. It should be noted that frozen sections are manipulated in the same way as Celloidin sections, but greater care must be exercised on account of the absence of any embedding mass.

Tissues should not be frozen too hard or the sections will curl up and split.

A special microtome is required for cutting frozen sections.

Rapid Method for Staining Fats

Solutions required:

A. Sodium chloride A.R. grade 0·9% in distilled water .. .. 95 ml.
Formalin .. .. 5 ml.

B. Sudan Black B, saturated in 70% alcohol (this should be freshly filtered).

NOTE: Ethylene or Propylene Glycol may be employed as the solvent (*See page 37*).

C. Apathy's mountant.

Technique:

1. Thin slices of tissue are fixed for ten minutes at 37–40° C. in Solution A.

2. Transfer the material directly from the fixative to the freezing microtome and cut sections about 5μ in thickness.

3. By means of a camel-hair brush, moistened with 50% alcohol, transfer sections from the microtome knife directly to the first of a series of dishes previously arranged as follows, in order of their use: 70% alcohol, Sudan Black solution (as above), 50% alcohol; distilled water.

4. After immersion in 70% alcohol (in the first dish) for two minutes, stain in the Sudan Black for ten minutes, or longer if time permits.

5. Rinse in the 50% alcohol.

6. Transfer to the distilled water: mount in Apathy's medium, or, if there is sufficient time, counterstain for about three minutes in Carmalum; then rinse in distilled water before mounting.

Results:

Neutral fat and myelin: blue-black to black; nuclei: red.

GELATINE EMBEDDING

This method of embedding is employed when sections of loose friable tissues are required. Dehydration is entirely eliminated since the embedding takes place directly from water. The gelatine which is retained during the staining processes holds the tissues together without absorbing the stain itself.

Solution required:

A. *Gelatine* 5%

Gelatine	5 gm.
Phenol 1% aqueous	95 ml.

Warm in an oven to about 45° C. then stir until the gelatine has dissolved; raise to about 60° C. before filtering through fine calico.

B. *Gelatine* 10%

Phenol 1% aqueous	90 ml.
Gelatine	10 gm.

Prepare exactly as for Solution A.

C. *Gelatine* 15%

Phenol 1% aqueous	85 ml.
Gelatine	15 gm.

Prepare exactly as for Solution A, except that temperature should be raised to about 75° C. before filtering.

D. *Gelatine* 20%

Gelatine	20 gm.
Phenol 1% aqueous	80 ml.

Prepare as for Solution A, but raise temperature to 95° C before filtering.

E. *Gelatine* 1%

Gelatine	1 gm.
Phenol 1% aqueous	99 ml.

Dissolve by warming.

Technique:

1. Small pieces of tissue, not more than 3 mm. in thickness are fixed for twenty-four hours in 5% formalin in physiological saline.

2. Wash in running water.

3. Immerse in 5% gelatine in an incubator at 37° C. for twenty-four hours.

4. Immerse in 10% gelatine overnight in an incubator at 37° C.

5. Immerse in 15% gelatine in an incubator for several hours at 37° C.

6. Embed in 20% gelatine and leave to set.

7. Cut out blocks of tissue and immerse them in formalin for twenty-four hours.

Note: The blocks may be stored indefinitely in this formalin solution if desired.

8. Rinse blocks in water and trim.

9. Freeze blocks thoroughly until they are uniformly white.

10. Allow the block to thaw somewhat until the knife cuts easily.

11. Cut sections up to 5μ in thickness and float onto slides with distilled water.

12. Drain off excess water and float sections on slides with two or three drops of 1% gelatine.

13. Drain off excess 1% gelatine and leave the slides in an oven at 37° C. until the sections are dry.

14. Immerse slide in 10% formalin for ten minutes to fix the gelatine; then stain in the usual manner with Sudan 3, or Scarlet R, Nile blue or osmic acid, or store the slides in the 10% formalin until required.

LOW VISCOSITY NITROCELLULOSE (L.V.N.)

For embedding tissues

The following technique, using L.V.N. in place of Celloidin, has been developed by E. H. Leach and W. Chesterman, of The University Laboratory of Physiology, Oxford.*

I am indebted to the authors and to the Oxford University Press for permission to print this description of the procedure.

Chesterman and Leach's technique using Low Viscosity Nitrocellulose (L.V.N.) offers advantages over the older method of embedding in Celloidin, in that penetration is quicker, considerably thinner sections can be cut, it is easier to use and considerably cheaper than Celloidin. With L.V.N. technique large blocks, such as half a cat's brain, can be cut at 15μ; small blocks 5 × 5 mm., can be cut at 5 to 7μ on a paraffin microtome without any special modification or attachment.

L.V.N. is supplied damped with normal butyl alcohol; it is more explosive than Celloidin and it should be handled with care. When dry it will explode if hit. Exposure to sunlight should be avoided.

Solutions required:

Note: Solutions A and B each contain 20% of Nitrocellulose:

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A. Absolute alcohol .. .. 210 ml.
Ether .. .. 250 ml.
Dibutyl phthalate .. .. 5 ml.*

Mix well and add 140 gm. L.V.N. (as supplied damped with N. butyl alcohol).

* NOTE

In the original paper (Chesterman and Leach, 1949) and in the previous edition of this book (1953), tricresyl phosphate was stipulated: this has now been replaced by dibutyl phthalate, and I am indebted to Professor F. Bergel of The Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, for calling my attention to the potential danger of handling tricresyl phosphate. Professor Bergel informs me that tricresyl-o-phosphate is highly toxic, more than 7–30 mg./kg. producing severe paralysis, and while the corresponding meta- and para-compounds show little sign of having this toxicity, the tricresyl phosphates on the market, up to the present time, always contain some of the ortho compound.

References: Aldridge, W. N. (1954), *Biochem. J.*, **56**, no. 2, 185–9, " Tricresyl Phosphates and Cholinesterase". Thompson, R. H. S. (1954), *Chem. and Ind.*, 749, *Martindale's Extra Pharmac.* (1941), vol. 1, 200–1.

B. Absolute alcohol .. .. 210 ml.
Ether .. .. 250 ml.

Mix well and add 140 gm. L.V.N. (damped with N. butyl alcohol).

From Solution B prepare also 5% and 10% solutions by diluting with a mixture consisting of equal parts of absolute alcohol and ether.

C. Xylol .. .. 2 parts
Toluol .. .. 1 part
Beechwood creosote .. .. 1 part

Procedure for embedding tissues:

1. Fix and dehydrate tissues as usual; then immerse in ether-absolute alcohol (equal parts) for one day.

2. Immerse in 5% L.V.N. for three to five days.

3. Transfer to 10% L.V.N. for one to two days.

4. Transfer to 20% L.V.N. (Solution B above) for one to five days.

5. Embed in Solution A.

6. Allow to harden slowly in a desiccator. In one to five days the block should be adequately hard. At this stage it should be a

stiff but easily deformable gel not altered in shape or size by shrinkage; it should be considerably less hard than a Celloidin block is usually made.

7. Plunge the block into 75% alcohol. Change the alcohol twice over a period of one to three days.

8. Trim the block, removing the hard outer rim of the L.V.N. Use 20% L.V.N. to mount it on the wood or fibre block. Hardening is complete in a few minutes. Dip into 75% alcohol for a few more minutes.

Cutting sections:

Cut the sections " dry ". If a Celloidin microtome is used the tilt of knife should be the same as that used for cutting Celloidin, but the angle the knife makes with the direction of travel should be between 25° and 45° instead of the usual 75° used for Celloidin sections; this prevents the rolling of sections.

Procedure for handling sections:

1. Collect the sections in 75% alcohol; handle and stain as usual. Dyes tend to stain L.V.N. less than Celloidin.

2. Mount sections on to a slide from a bowl of 96% alcohol. Flatten with tissue paper moistened with 96% alcohol; press the paper with a glass rod and then remove it. Repeat this several times.

3. Treat similarly several times with equal parts of absolute alcohol and chloroform.

4. Treat similarly several times with the solution C.

5. Treat similarly several times with xylol.

6. Mount in balsam.

MERCURIC CHLORIDE PRECIPITATES IN SECTIONS: METHOD FOR REMOVAL

Before proceeding to the staining of sections of tissues which have been fixed in fluids containing mercuric chloride it is necessary to carry out the following procedure, which is essential for the removal of the deposits of mercuric chloride which would obscure the picture.

Solutions:

A.	Iodine	0·5 gm.
	Alcohol 70%	100 ml.
B.	Sodium hyposulphite	7·5 gm.
	Alcohol 96%	100 ml.
	Distilled water	900 ml.

A crystal of thymol should be added to the stock bottle.

Technique:

1. Sections are mounted on slides and the paraffin wax removed with xylol. If there is any doubt as to the nature of the fixative which has been used, examine a section under the microscope: mercuric chloride, if present, will be seen as a fine brown granular deposit, more abundant in the centre of the section than on the outer edges.

2. Wash with absolute, followed by 90% alcohol.

3. Immerse for half to two minutes in Solution A.

4. Wash well with water.

5. Immerse in Solution B for half to two minutes or until the natural colour of the sections has been restored.

6. Rinse well with water.

7. Stain, dehydrate and clear in the usual manner.

PARAFFIN WAX EMBEDDING AND SECTIONING

(*a*) Dehydration

Technique:

1. Pieces of tissue are fixed and washed by any of the standard methods.

2. Immerse in 50% alcohol from twelve to twenty-four hours.

3. Transfer to 70% alcohol for the same length of time as stage 2.

4. Transfer to 90% alcohol for the same length of time.

5. Transfer to 96% alcohol for the same length of time.

6. Immerse in absolute alcohol for the same length of time.

Note: Where possible use a series of corked specimen tubes for the above procedures. By occasionally shaking the tube containing the specimen the process of penetration of the alcohol is accelerated. Twelve hours in each change of alcohol is sufficient for small pieces, but larger pieces of tissue usually require eighteen or twenty-four hours. Hard tissues may be softened by Lendrum's technique which consists of immersing the tissues in 4% aqueous phenol for one to three days, after washing out the fixative; dehydration is then carried out in the manner described above.

Rapid dehydration of small slices of tissue:

Thin slices not more than 5 mm. in thickness are immersed for half an hour in each of 50%, 70%, 90%, 96% and absolute alcohol.

(*b*) CLEARING

Xylol, cedarwood oil, benzene, toluene or chloroform are the reagents most frequently used for this purpose.

Xylol is the most rapid of these in displacing the absolute alcohol, but it has the disadvantage of rendering tissues brittle; therefore, if xylol is used as the clearing agent tissues must be subjected to it only for the minimum time necessary to displace the absolute alcohol.

Cedarwood oil is slow in its action but it has the advantage of not hardening tissues even after prolonged immersion.

Benzene is the best clearing agent and may be employed for the most delicate tissues: it causes the minimum shrinkage, penetrates tissues fairly rapidly and subsequently evaporates from them in the paraffin embedding bath.

Toluene is also a very satisfactory clearing agent in that tissues can be subjected to it for at least twenty-four hours without risk of their undergoing shrinkage.

Chloroform hardens tissues to a lesser degree than xylol but requires two or three times as long to penetrate and clear the tissue. It rapidly evaporates from the paraffin embedding bath, and it is particularly suitable for large pieces of pathological tissue.

Technique:

1. Small pieces of material not more than 5 mm. thick may be cleared by immersing for fifteen to thirty minutes in each of two

changes of xylol or cedarwood oil or benzene or toluene. Larger pieces up to 1 cm. thick require one-and-a-half to three hours in each of two changes of the clearing agent, while bulky specimens such as whole embryos require up to six hours in each of the two changes. If at the end of the times prescribed above the specimens are not translucent or transparent they should be left in the clearing agent until they have reached that stage.

(*c*) Embedding

Technique:

1. Transfer the object from the clearing agent to a mixture consisting of approximately equal parts of paraffin wax and the clearing agent in a tube and place the whole in the oven set at a temperature from about 50 to 60° C. for one half to sixteen hours, depending upon the size and nature of the object; half an hour is sufficient for objects up to 3 mm. thick; an hour for 5 mm., two hours for 1 cm., and from eight to sixteen hours for bulky specimens such as whole embryos.

2. Transfer to pure paraffin wax in the oven from a quarter to eight hours.

3. Transfer to another bath of pure paraffin wax for the same length of time.

Note: Specimens up to 3 mm. thick usually require half an hour in each of the two baths of pure paraffin wax, while specimens 5 mm. in thickness require about an hour; and 1 cm. about four hours; very bulky objects about eight hours in each of the two baths of wax.

Pathological material containing thrombi, emboli, etc., striated and non-striated muscle, organs containing a large amount of blood (spleen, etc.), and fibrous tissue should be subjected to immersion in the embedding baths for the minimum time necessary for the wax to penetrate thoroughly, as they are particularly liable to hardening and shrinkage when exposed to heat for prolonged periods.

Casting the Paraffin Block

1. Smear the inside of the embedding angles and the embedding base-plate very thinly with liquid paraffin; then adjust the angles on the plate to form a mould of a suitable size.

2. Fill the mould with molten paraffin wax; then place the object in the wax and arrange it so that it is set in the right plane for sectioning.

3. When the wax block so formed is partially set, immerse it gently, while still in the mould, in cold water to ensure rapid cooling and thereby obviating crystallization of the wax and consequent crumbling of the block when it is mounted on the microtome and sections are cut.

Cutting of Sections

This can only be learnt by practical experience under skilled guidance in the laboratory, and it is not proposed to make any attempt to deal with the subject here since space does not permit a description of the various types of microtomes available and the technique of manipulating them. However, readers would find the chapter on section cutting in *Histological Technique*, by H. M. Carleton and E. H. Leach (published by Oxford University Press), very informative.

Mounting Sections on Slides and Hydrating

1. Wet the tip of the finger slightly with glycerine albumen (Mayer) and make a smear over an area large enough to take the section in the centre of the slide.

2. Pick a section up with a needle or forceps and place it over the smear of albumen.

3. With the thumb, gently press the section on to the smear so that it is quite flat and without folds or creases, taking care not to damage the section in the process.

Note: If the sections are curled up or folded, first place a drop of 1% potassium dichromate on the slide and float the section on this; then heat very gently until the section floats out flat. Blot round the edges of the section to remove excess solution; then carefully but thoroughly blot until all traces of liquid are removed. Leave the slide on a warm surface for a few minutes to drive away the last traces of water; then proceed as follows:

4. Gently warm the slide until the paraffin wax just melts; then wash away all traces of wax with two or three changes of xylol.

5. Remove the xylol by washing the preparation thoroughly with absolute alcohol.

6. Wash with two changes of 90% alcohol.
7. Wash with 70% alcohol.
8. Wash with two changes of distilled water if an aqueous stain is to be used; but if an alcoholic stain will be used staining may commence immediately after washing with 70% alcohol.
9. Proceed to stain in accordance with the staining technique it is desired to employ.

Note: If the section appears opalescent when the xylol or when the absolute or the 90% alcohol is added the presence of water is indicated and it is necessary to retrace each step until the preparation no longer appears opalescent when taken down to alcohol.

PARAFFIN WAX - PYRIDIN TECHNIQUE

A rapid method of dehydrating and clearing

1. Material is fixed and washed by the standard method.
2. If a fixative containing mercury has been used, remove mercurial precipitate by the standard technique.
3. Immerse the tissue in two changes of Pyridin for two to eight hours in each, according to the nature and thickness of the tissue.
4. Transfer to a mixture of equal parts of molten paraffin wax and Pyridin in the embedding oven for one half to sixteen hours, depending upon the size and nature of the object.
5. Transfer to pure paraffin wax in the oven for a quarter to eight hours, depending upon the thickness and nature of the material.
6. Cast into block and cut sections in the usual manner.

WATER WAX

(Michrome)

A very rapid and simple method of embedding tissues, obviating the use of dehydrating and clearing agents.

Water wax is an amorphous water-soluble wax which sets at 56° C. to form translucent blocks similar in appearance to paraffin wax but with the complete absence of any trace of crystallization. Fresh or fixed material may be used.

Reagent required:

Water wax (Michrome).

Technique:

1. Shake off excess water before immersing the pieces of tissue directly into a bath of water wax in the embedding oven at 55–6° C. and leave for an hour.
2. Transfer to a second bath of water wax and leave for an hour.
3. Transfer to a third bath of wax for an hour, or longer.
4. Cast the block and leave it to cool in the atmosphere or in a refrigerator. Care must be taken not to allow the block to come into contact with water.
5. After cutting sections, float them out on water, which dissolves away the wax, and take them up on slides.
6. Stain, dehydrate and mount immediately in the usual way.

Notes:

(*a*) Fat, if present, should be dissolved out with several changes of acetone, before the tissues are immersed in the wax.

(*b*) Blocks should be kept dry and stored in airtight containers as the wax is liable to take up moisture from the atmosphere.

MISCELLANEOUS DEHYDRATING AND CLEARING REAGENTS

BUTYL ALCOHOL TERTIARY

For dehydrating and clearing tissues for paraffin embedding in place of ethyl alcohol and xylol. The reagent is miscible with water and with paraffin wax, and causes less shrinkage and hardening of tissue than does ethyl alcohol and xylol. It is also a useful substitute for ethyl alcohol for dehydrating material stained with methylene blue and other dyes which are easily extracted by ethyl alcohol.

Technique:

After fixing and washing tissues in the usual manner pass into:

1. Tertiary butyl alcohol (T.B.A.) 50% aqueous for 1–2 hours.
2. 70% Aqueous T.B.A. 2 hours to several days.
3. 85% aqueous T.B.A. for 1–2 hours.

4. 95% aqueous T.B.A. for 1–3 hours.
5. Pure T.B.A. for 3 changes of 4 hours in each.
6. Equal parts of liquid paraffin and T.B.A. for 1–2 hours.
7. Infiltrate in paraffin wax.

CAJEPUT OIL

For clearing

This reagent will absorb small amounts of water without clouding, and it is, therefore, particularly useful in wet climates as a clearing agent in place of xylol. Cajeput oil is considerably more expensive than xylol, however.

CELLOSOLVE

(Ethylene glycol monethyl ether)

For dehydrating thin slices of tissue and sections

This reagent, which is a colourless, inflammable liquid, miscible with water, alcohol, xylol, cedarwood oil, clove oil, and various other oils and solvents, and is also a good solvent for many stains, is coming into increasing use both for animal and plant histology, in place of ethyl alcohol: in fact many laboratories in Great Britain, at least, employ cellosolve in preference to the ethyl alcohol technique, which they now regard as obsolete. However, although some workers believe cellosolve to be superior to all other dehydrating agents as it obviates hardening and distortion of most tissues, it is unsuitable for bulk material as it tends to cause distortion of protoplasmic cells owing to the rapidity of its dehydrating action.

Technique:

1. Wash pieces of tissue, not more than 5 mm. thick and immerse directly into cellosolve for half an hour.
2. Immerse in a fresh bath of cellosolve for half to one hour.
3. Immerse in a third bath of cellosolve for the same time.
4. Complete the dehydration in a fourth bath of cellosolve for an hour-and-a-half.

5. Clear in xylol for an hour.

6. Immerse in a bath of molten paraffin wax for half an hour.

7. Transfer to a second bath of paraffin wax for an hour.

8. Complete the infiltration by immersing in a third bath of wax for an hour; then cast the block and cut sections.

9. Fix sections to slides; then remove paraffin wax with xylol.

10. Rinse in two changes of cellosolve.

11. Apply the stain; then wash with water, or alcohol.

12. Immerse for one to three minutes in each of three changes of cellosolve.

13. Clear in xylol, benzol, clove oil or cedarwood oil.

14. Mount in balsam, cristalite or D.P.X. (Lendrum and Kirkpatrick).

DIOXANE

For the dehydration and clearing of tissues

This reagent, which is a colourless inflammable liquid, solidifying at 10° C., miscible with water and paraffin wax, alcohol and xylol as well as many other organic solvents of the aliphatic and of the aromatic series, is used and preferred by many workers in place of the orthodox alcohol-xylol-cedarwood oil method for dehydrating and clearing tissues, as the technique is simpler and quicker and it has the advantage of eliminating brittleness and shrinkage of tissues. It is, however, essential that a reliable brand of Dioxane be employed, as some makes contain relatively large amounts of water, and are therefore unsuitable for histology.

Warning.—Care should be taken as Dioxane vapour is toxic: it should be used only where ventilation is abundant.

Technique:

1. Transfer tissues directly from the fixative to a well-stoppered specimen jar containing Dioxane with a thin layer of anhydrous calcium chloride over which is placed a circle of surgical or zinc gauze to separate the tissue from the dehydrating agent. Allow the Dioxane to act from three to twenty-four hours, depending upon the size and thickness of the tissue.

Note: Tissues which have been treated with a fixative such as Müller or Zenker, containing potass. dichromate, must be washed from two to twelve hours in running water, depending upon the size and the nature of the tissue, before being transferred to Dioxane.

2. Transfer to a mixture of equal parts of paraffin wax and Dioxane for half to one hour in a paraffin embedding oven.

3. Transfer to pure paraffin wax, allowing a somewhat longer time for impregnation than for tissues cleared by the orthodox method.

Note: The Dioxane can be used several times provided the calcium chloride is changed.

ETHYLENE GLYCOL

A solvent, inflammable when heated, burning with an intense, almost invisible flame; which may also be employed as a dehydrating and differentiating agent for the Sudan colours, in place of acetone-alcohol, 70% or 50% alcohol; giving stable solutions, without loss of stain from the lipid particles.

Solution required

Technique:

1. Prepare the staining solution by heating and stirring about 0·75 gm. of the dye with 100 ml. pure anhydrous ethylene glycol to 100° C. Filter the solution while it is still hot, and again after it has been allowed to cool.

2. Cut frozen sections, from formalin fixed material, and wash them in water for about five minutes to remove excess formalin.

3. Dehydrate the sections by agitating them gently with a camel hair brush for three minutes in each of two changes of pure anhydrous ethylene glycol.

4. Transfer the sections to the staining solution for five to seven minutes, agitating them gently at intervals.

5. Differentiate by agitating the sections gently in 85% ethylene glycol in water for two to five minutes, controlling by examining under the microscope at intervals.

6. Transfer to a large dish of distilled water for three to five minutes.

7. Float sections onto slides; drain and carefully blot away excess water.

8. Mount in glycerine jelly, Farrant or Aquamount.

Note: Either ethylene or propylene glycol may be used; however, the former usually costs less than the latter.

Reference: Chiffelle, T. L. and Putt, F. A., (1951), *Stain Tech.*, **26**, 51–6.

PROPYL ALCOHOL, NORMAL (OR ISO)

For dehydrating and clearing tissues prior to embedding

Technique:

1. Pieces of fixed tissue are placed directly into normal propyl alcohol and left therein overnight.

2. Transfer directly into a bath of paraffin wax M.P. 40° C.

3. After infiltration of the 40° C. wax, transfer to a bath of 52–54° C. paraffin wax for a few minutes; then cast the block.

Note: This method prevents hardening and distortion of tissue: it is particularly recommended for scirrhous carcinoma, connective tissue, tumours, etc.

TERPINEOL

For dehydrating sections

Technique:

1. After staining and before dehydrating, wipe the slides and blot sections carefully, without allowing them to dry completely.

2. Transfer to terpineol and agitate for a few seconds.

3. Immerse in a second lot of terpineol for a few seconds.

4. Drain and wipe the slides carefully.

5. Clear with xylol, and mount as usual.

Note: The destaining action of alcohol is avoided with this method, which is harmless to the vast majority of stains, Neutral Red being an exception. If desired, Cajeput Oil may be used for clearing.

AQUEOUS MOUNTING MEDIA

APATHY GUM SYRUP MOUNTANT

A fairly quick-drying aqueous mountant which sets very hard and may be used in place of Farrant for fat preparations; it is, however, usually definitely acid in reaction. Hardening of the mountant may be hastened if the slides are left on warm plate. Apathy may also be used for ringing glycerine mounts.

AQUAMOUNT

A moderately quick-drying, transparent and colourless, aqueous mountant which, unlike Apathy and Farrant, is neutral in reaction. It takes somewhat longer to harden than does Apathy but it is unlike the latter in that it does not tend to crystallize or become excessively brittle. Aquamount is preferable to Apathy or Farrant for fat preparations.

BERLEZE'S FLUID

A mountant and killing fluid used in Entomology. Living specimens of Acarina and Insecta are killed by placing them directly into a drop of this fluid on the slide, but specimens which have been stored in alcohol should be washed with 10% aqueous acetic acid before mounting. The fluid takes from one to two weeks or even longer to set, after which time the slides should be ringed with a waterproof cement and ringed with a layer of Canada balsam in benzene.

DOETSCHMAN'S GUM CHLORAL MOUNTANT AND STAIN

This is a modification, containing basic fuchsin, of Berleze's fluid, which kills, fixes, dehydrates, clears, stains and mounts a specimen in one operation.

This valuable reagent is used in entomology for killing, fixing, dehydrating, clearing, staining and mounting specimens in one operation. Highly chitinized specimens should be treated with 10% potassium hydroxide from sixteen to twenty-four hours, then washed well in water before mounting directly into a drop

of Doetschman's fluid on a slide. For permanent mounts the preparations should be placed in the oven at 37–50° C. from six to twenty-four hours. Ring the slides with a waterproof cement.

Reference: *Microscopist's Vade-Mecum* 11th ed., p. 207.

GLYCERINE

Used as a mounting medium for frozen sections. Only the purest neutral glycerine should be used. Permanent preparation may be made by painting the edges of the coverslips with melted glycerine jelly and coating the jelly, when it has set, with gold size or asphalt varnish.

GLYCERINE JELLY

For fat preparations, frozen sections, gelatine sections, etc. This mountant, which should be neutral in reaction, sets sufficiently hard to permit direct varnishing of the edges of the coverslips, of mounted preparations, without prior luting. Preparations mounted in glycerine jelly will last for many years without deterioration.

GLYCHROGEL MOUNTANT

For Marchi-stained sections, gelatine sections, teased preparations, nematodes etc.

A.	Chrome alum	0·2 gm.
	Distilled water	30 ml.

Dissolve by warming.

B.	Gelatine granules	3 gm.
	Distilled water	50 ml.
	Glycerine	20 ml.

Warm the water to 45° C. then shake in the gelatine a little at a time until dissolved and add the glycerine.

Add solution A to solution B; shake thoroughly; then filter and add about 0·1 gm. camphor as a preservative.

Keep in a well-closed bottle to prevent evaporation.

Reference: Wotton, R. M. and Zwemer, R. L. (1935), *Stain Tech.*, **10**, no. 1, 21–2.

NON-AQUEOUS MOUNTING MDEIA

CANADA BALSAM

Strictly speaking " Canada balsam " is natural Canada balsam, a pale-yellow viscous fluid obtained from the balsam fir, indigenous to Canada: this fluid is useless as a histological mounting medium. "Canada balsam, dried " is the natural Canada balsam which has been dried by heat until it has become a brittle solid.

" Canada balsam in xylol " (or in benzene or other suitable solvents) is prepared by dissolving the dried natural balsam in xylol, etc. When an histologist sees the words " Mount in Canada balsam " (or simply " balsam ") he takes it to mean, and correctly so, that the preparation is to be mounted in Canada balsam in xylol. However, the term " Canada balsam " can be misleading to those who are new to the language and customs of histology, and such workers have been known to mount in true Canada balsam: i.e. natural Canada balsam, and with most unsatisfactory results. It is for this reason that this definition of " Canada balsam " is given here.

Although Canada balsam in xylol is still used very extensively it has a serious disadvantage in that it is prepared from a natural resin which is uncertain and variable in chemical composition, usually somewhat acid in reaction, and while it may be neutralized, will revert to an acid reaction after a time, however careful one is in handling and storing the mountant. For this reason it is better to employ a synthetic mountant of definite and unvarying chemical composition. Short descriptions of mountants of this kind are given below: they also have the advantage of being considerably less costly than Canada balsam, moreover, Canada balsam is considerably more costly than the synthetic mounting media described below.

CLEARMOUNT

Refractive index 1·515

A colourless, neutral, synthetic mountant, with a drying time approximately the same as Canada balsam in xylol. This mountant, which remains neutral indefinitely and does not cause the

fading of even the most delicate stains, is miscible with xylol, absolute alcohol, benzene, toluol, dioxane and many other solvents.

CRISTALITE

Refractive index 1·515

This synthetic mountant has all the properties of Clearmount except that it dries much more rapidly and is not miscible with alcohol.

D.P.X.

Refractive index 1·515

A synthetic mountant, devised by Lendrum and Kirkpatrick* on which Cristalite and probably other proprietary synthetic mountants are based. This excellent mountant, which is colourless and neutral and does not cause fading of stains has the added advantage of being one of the least costly of all the synthetic resinous mountants: however, there is a considerable degree of shrinkage of the mountant on drying and it should therefore be applied liberally to allow for this.

* *J. Path. Bact.*, (1939), **49**, 592; (1941), **53**, 441.

MEEDOL BALSAM

Refractive index 1·515

A xylol miscible mountant, pale amber in colour and hardly distinguishable in appearance from Canada balsam in xylol. This is one of the least costly of all resinous synthetic mounting media. Unlike Canada balsam, but like the other synthetic mountants described in this paragraph, Meedol balsam has the advantage of remaining neutral indefinitely under normal conditions of storage and handling.

FLUORMOUNT

Refractive index 1·515

A synthetic, colourless, neutral, xylol miscible, non-fluorescent mountant for fluorescence microscopy.

MICHROME MOUNTANT

Refractive index 1·515

A synthetic neutral mountant miscible with alcohol and with xylol. Sections may be mounted directly from 95% alcohol if desired. Does not cause the fading of stains even after several years. This mountant, however, is largely being displaced by Clearmount.

S.Q.D. BALSAM

Refractive index 1·515

An exceptionally quick-drying, synthetic, xylol miscible, synthetic mountant, similar to Meedol balsam.

VENETIAN TURPENTINE

For mounting filamentous algae and other delicate material

Solutions required:

A. Glycerin 5%.
B. Venetian turpentine 10% in absolute alcohol.

Technique:

1. Stain the material in suitable aqueous stains.

2. Transfer to Solution A, and leave therein for several days in an open dish.

3. Wash with several changes of 95% alcohol.

4. Wash with two or three changes of absolute alcohol.

5. Transfer the material quickly from absolute alcohol to Solution B (10% Venetian turpentine in absolute alcohol), in an open dish.

6. Place the dish with contents over soda lime in a desiccator for several days until the fluid becomes viscous.

7. Mount on slides.

SECTION 2—ANIMAL HISTOLOGY

(Normal and Pathological)

ALCIAN BLUE - CHLORANTINE FAST RED

For selective staining of mucopolysaccharides and for morphological studies of connective tissue, cartilage and bone

Solutions required:

A. Ehrlich haematoxylin

B. Alcian blue 1% aqueous .. .. 50 ml.
Acetic acid 1%, aqueous .. .. 50 ml.

C. Phosphomolybdic acid 1% aqueous

D. Chlorantine fast red 0·5%, aqueous

Technique:

1. Fix tissues in Bouin or in 10% formalin and embed in the usual way.

2. Stain sections in Ehrlich haematoxylin for ten to fifteen minutes.

3. Blue in tap water or in lithium carbonate solution.

4. Wash in distilled water.

5. Stain for ten minutes in the Alcian blue.

6. Wash in distilled water.

7. Immerse in the phosphomolybdic acid solution for ten minutes.

8. Wash in distilled water.

9. Stain for ten minutes in the chlorantine fast red solution.

10. Wash in distilled water.

11. Dehydrate; clear in xylol and mount.

Results:

Nuclei are stained purplish blue. Mucin, granules of mast cells, ground substance of cartilage and some types of connective tissue

fibres, bluish green. Collagen fibres and ossein, cherry red. Cytoplasm and muscle, pale yellow.

Reference: Lison, L. (1954), *Stain Tech.*, **29**, no. 3, 131–8.

ALDEHYDE FUCHSIN (G. Gomori)

For elastic tissue, mast cells, beta cells of the pancreatic islets, etc.

Solutions required:

A.	Alcohol 70%	100 ml.
	Iodine crystals	0·5 gm.
B.	Sodium thiosulphate	0·75 gm.
	Alcohol 96%	10 ml.
	Distilled water	90 ml.
C.	Basic fuchsin	0·5 gm.
	Alcohol 70%	100 ml.
	Hydrochloric acid, conc.	1 ml.
	Paraldehyde	1 ml.

Dissolve the basic fuchsin in the alcohol; to the cold solution add the acid and the paraldehyde; shake well; then leave to stand for about twenty-four hours until the colour of the solution has changed to violet (almost indistinguishable in appearance from gentian violet). As soon as this change has taken place the stain is ready for use.

Note: The solution will keep for about four weeks at room temperature, but as the stain ages during the four weeks, longer staining times are necessary.

Technique:

1. Tissues may be fixed in almost any fixative, but those containing dichromate are not recommended as they adversely effect the clearness of the final picture.

2. Embed in paraffin wax for preference: if celloidin is used, the celloidin must be removed completely from the sections before staining as it is impervious to the stain.

3. Fix sections to slides and remove paraffin wax in the usual way.

4. Wash with absolute alcohol followed by 90% alcohol.

5. Immerse in solution A in a stoppered jar for ten minutes to one hour.

Note: This treatment with iodine is recommended for all tissues, whether they have been fixed in mercurial fixatives or not, as it often shortens the staining time necessary and makes the shade deeper.

6. Wash well with water.

7. Immerse in solution B for about one half to two minutes until the natural colour of the section has been restored.

8. Wash well with water.

9. Stain for five minutes to two hours in a coplin jar filled with solution C.

Note: Elastic fibres, five to ten minutes. Beta cells, fifteen to thirty minutes, or longer. Pituitary, thirty minutes to two hours.

10. At intervals examine the slide, after rinsing with 90% alcohol, under the microscope to ascertain the depth of staining, but taking care that the preparation is not allowed to dry. If the desired depth of staining has not been attained, the slide may be returned to the stain and rinsed with alcohol again before further examination: this process may be repeated any number of times until the desired degree of staining has been reached.

11. If desired a counterstain may now be applied: haematoxylin - Orange G is best for most purposes, but for pancreas and pituitary, a trichrome stain of the Masson type or the Mallory-Heidenhain technique can be used to bring out all types of cells. In either case, Light Green or Fast Green, FCF should be used in place of the aniline blue as their shades contrast better with the purple of the aldehyde fuchsin.

12. Dehydrate with absolute alcohol; clear in xylol and mount.

Results:

The following are stained deep purple:

(I) Elastic fibres of all tissues, whatever fixative has been used.

(II) Mast cells, after any fixative.

(III) The chief cells of the gastric mucosa, particularly well stained after fixation in formalin or Bouin.

(IV) Beta cells of the pancreatic islets of all species, after formalin, mercuric chloride-formalin or Bouin. Particular beautiful results are obtained in the islets of man and the sheep (the beta cells of the latter are particularly difficult to stain otherwise).

(V) Functioning tumours of the islets are also stained selectively.

(VI) Certain basophils of the anterior pituitary, after the same fixatives as in (V). In the pituitary of the rat and of the pig the two kinds of basophils are usually quite conspicuous.

Notes:

(*a*) After fixatives containing mercury the background is pale mauve. After formalin or Bouin the background is colourless.

(*b*) If the stain is prepared by adding paraldehyde to Feulgen's fuchsin the resultant solution will stain the beta cells very distinctly but leave the elastic fibres unstained. Old solutions of the aldehyde fuchsin will stain elastic fibres very selectively, but leave the beta cells unstained.

Reference: Gomori, G. (1950), *Am. J. Clin. Path.*, 20, No. 7, 665–6.

ALDEHYDE FUCHSIN - HAEMATOXYLIN LIGHT GREEN - ORANGE G - CHROMOTROPE

For the differentiation of two types of Basophils in the Adenohypophysis of the rat and the mouse

Solutions required:

A. Bouin's fixative with the acetic acid replaced by 0·5% trichloracetic acid.

B. Lugol's Iodine

C. Sodium thiosulphate 5% aqueous

D. *Aldehyde fuchsin*

Basic fuchsin	0·5 gm.
Absolute alcohol	60 ml.
Distilled water	40 ml.
Paraldehyde	1 ml.
Hydrochloric acid, conc.	1·5 ml.

Note: The solution turns purple in 24 hours, and is ripe and ready for use after being kept at 20° C. for three days or in two days if kept at about 37° C. The stain deteriorates after four or five days.

E. Ehrlich Haematoxylin

F. Ethyl alcohol 70% .. .. .. 99·5 ml.
Hydrochloric acid, conc. .. .. 0·5 ml.

G. Lithium carbonate, saturated aqueous

H. Orange G 2% aqueous .. .. 50 ml.
Light Green SF 1% aqueous .. 20 ml.
Distilled water .. .. .. 30 ml.
Chromotrope 2R .. .. .. 0·5 gm.
Phosphotungstic acid .. .. 0·5 gm.
Glacial acetic acid .. .. .. 1 ml.

Dissolve the phosphotungstic acid in the distilled water, then add and dissolve the chromotrope 2R followed by the acetic acid, Orange G and Light green solutions. Shake thoroughly.

Note: This solution keeps indefinitely.

I. Acetic Acid 0·2%

Technique:

1. Fix in solution A for 24 hours.

2. Wash in running tap water for six to eight hours.

3. Dehydrate, clear and embed in paraffin wax in the usual manner.

4. Cut sections, in the horizontal plane, 3 to 4μ in thickness.

5. Remove paraffin wax from sections with xylol.

6. Pass through the usual descending grades of alcohol to distilled water.

7. Immerse in Lugol's iodine for thirty minutes.

8. Transfer to the sodium thiosulphate solution until the sections have regained their natural colour (about two minutes).

9. Rinse thoroughly in distilled water.

10. Stain in the aldehyde-fuchsin solution from two to ten minutes, taking care not to overstain, which can be avoided by checking the slides (after rinsing in 95% alcohol) at intervals under the microscope: the staining should be stopped as soon as the beta cells stand out clearly in dark purple against a colourless or faintly purple background.

11. Rinse in two changes of 95% alcohol.

12. Immerse for five to ten minutes in a third change of 95% alcohol.

13. Rinse in 70% alcohol.

14. Rinse in distilled water.

15. Stain in Ehrlich Haematoxylin for three to four minutes.

16. Rinse in distilled water.

17. Differentiate by dipping three or four times in the acid alcohol solution F.

18. Blue in the lithium carbonate solution; or in running tap water for five to ten minutes.

19. Counterstain in the light green-orange-chromotrope solution H for 45 seconds.

20. Rinse quickly with 0·2% acetic acid.

21. Rinse in 95% alcohol.

22. Immerse for two minutes in each of two changes of absolute alcohol.

23. Blot slides carefully.

24. Immerse in xylol for two minutes.

25. Immerse in another lot of xylol for five minutes.

26. Mount in D.P.X. or Clearmount.

Results:

Granulation of beta cells, selectively stained dark purple with the aldehyde-fuchsin. The cells of the pas intermedia and the Herring bodies of the neutral lobe should have little or no affinity for aldehyde-fuchsin. The delta cells are stained green, and the acidophilic granules varying shades of orange. Nuclear chromatin, purplish brown to reddish brown. Nucleoli are tinged bright

red, by the chromotrope 2R. The non-granular cytoplasm, greyish green or unstained. Coagulated contents of the cytoplasmic vacuoles, orange.

Reference: Halmi, Nicholas, S. (1952), *Stain Tech.*, **27**, no. 1, 61.

ALIZARIN RED, S

For calcium deposits in cartilagenous and embryonic bone

Solutions required:

A. Alizarin Red, S, aqueous 1%
B. Polychrome Methylene Blue (Unna)

Technique:

Tissues are fixed in 80–90% alcohol and embedded in paraffin wax.

1. Sections are brought down to distilled water; then stained in Solution A for five to sixty minutes, according to the material.
2. Wash with distilled water, followed by 95% alcohol at 60° C.
3. Counterstain with Solution B for one to three minutes.

Results:

Cartilage: intense violet. Calcium: red. Nuclei: blue. Cytoplasm, etc.: yellow.

The method is particularly suitable for pathological specimens.

For bone staining in small vertebrates (Dawson's method)

Solutions required:

A. Potass. hydroxide 1% aqueous
B. Alizarin Red, S .. .. .. 0·1 gm.
Potass. hydroxide .. .. 10 gm.
Distilled water .. .. .. 1 litre
C. *Mall's solution:*
Glycerin .. .. .. 20 ml.
Distilled water .. .. 79 ml.
Potass. hydroxide .. .. 1 gm.

Technique:

1. Whole specimens are fixed in 95% alcohol for at least three days.

2. Transfer to acetone and leave for several days to dissolve out the fats which would otherwise stain intensely and obscure the view of the bony structures.

3. Wash well with 95% alcohol; then immerse in 95% alcohol for twenty-four hours.

4. Immerse in Solution A from one to seven days, according to the size of the specimen, until the bones are clearly visible through the muscle.

5. Transfer to Solution B until the bones are stained the desired depth of colour; this takes from one to seven days, and the solution should be changed on the fourth day.

6. Clear in Solution C until no more colour comes out.

7. Pass into a mixture of equal parts of glycerin and water, and continue through increasing strengths of glycerin.

8. Store in pure glycerin.

Results:

Bones are stained red; soft tissue, transparent and unstained.

Notes:

If the initial clearing in potass. hydroxide solution has progressed to the proper stage only the bone will be stained, but otherwise soft tissue will also be stained.

The prolonged preliminary fixation in alcohol renders the tissue less liable to maceration in the potass. hydroxide solution.

Objects fixed in liquids other than alcohol may be stained by this method provided they are soaked in 90% alcohol for at least three days. The best preparations are made with fish, but amphibia and mammals have also been tried with a fair degree of success, although there is not the same firm consistency about the flesh of a mammal or amphibian, prepared by this technique, as there is with that of a fish.

The technique is particularly suitable for demonstrating developing bone.

William's modification of Dawson's method

This technique is particularly suitable for mammalian embryos and mature specimens of Urodele amphibians; for distinguishing

between bone and cartilage and for demonstrating the relative amount of ossification.

The removal of the viscera is unnecessary in the case of museum specimens.

Solutions required:

A. Toluidine Blue .. .. .. 0·25 gm.
Alcohol 70% .. .. .. 100 ml.
Hydrochloric acid 0·5% .. 2 ml.
Allow the solution to stand for twenty-four hours; then filter and store in a tightly corked bottle.

B. Potass. hydroxide 2% aqueous ..

C. Alizarin Red, S .. .. .. 0·001 gm.
Potass hydroxide 2% aqueous .. 100 ml.
(This solution should be freshly prepared.)

D. Methyl salicylate 25% in cellosolve

E. Methyl salicylate 50% in cellosolve

F. Methyl salicylate 75% in cellosolve

Technique:

1. Wash specimens for twenty-four hours in 70% alcohol containing 0·2% of concentrated ammonia solution.

2. Stain for seven days in Solution A.

3. Harden and destain for seventy-two hours in four changes of 95% alcohol.

4. Macerate for five to seven days, depending on the size of the animal, in several changes of 2% aqueous potass. hydroxide. (*Note:* This process is hastened by exposure to sunlight.)

5. Transfer to Solution C for about twenty-four hours when the bones should be well stained. If the specimen has been insufficiently macerated the soft tissue will be slightly stained, in which case the specimen may be destained rapidly in acid alcohol (1% sulphuric acid in 95% alcohol).

6. Dehydrate by leaving the specimen in three changes of cellosolve for six hours in each. Instead of cellosolve, 50%, 80% and 90% alcohol, followed by three changes of benzol may be used for dehydration. Small embryos require less time in the dehydrating fluids.

7. Clear by transferring to solutions of 25%, 50% and 75% methyl salicylate in cellosolve for twenty-four hours in each.

8. Store in methyl salicylate.

Note: If glycerin is used for clearing the technique has to be modified as follows:

Omit stage 6 and transfer directly from the Alizarin Red S solution into a series of 50%, 70% and 80% glycerin for twenty-four hours in each; then store in pure glycerin.

Results:

Soft tissues: transparent. Osseous tissue: deep blue. Cartilage: dark blue.

Note: The relative degree of ossification and chondrogenesis which has taken place is indicated by the intensity of the stains. Bone and cartilage may be stained separately by omitting stage 2, or stage 5 for cartilage.

For foetal specimens

The technique is particularly suitable for mammalian embryos, for demonstrating minute bones and foetal ossification.

Solutions:

A.	Alizarin Red, S, 1% aqueous (freshly prepared)	1 litre
	Potass. hydroxide 1% aqueous ..	1 ml.
B.	Potass. hydroxide	10 gm.
	Water	800 ml.
	Glycerin	200 ml.

Note: For small specimens 5 gm. potass. hydroxide is sufficient.

Technique:

Fix in 95% alcohol for at least two weeks after making a midline abdominal incision to allow penetration of the fixative.

1. Rinse in tap water.

2. Immerse for at least four weeks in 1% potass. carbonate.

3. Immerse for at least ten days in 1% aqueous potass. hydroxide until the bones are clearly visible through the soft tissue.

Note: Formalin-fixed specimens require four to six weeks in 1% potass. hydroxide. Should the tissue become too soft it may be hardened by immersing for twelve to twenty-four hours in a mixture consisting of equal volumes of glycerin, water and 95% alcohol before returning to the clearing solution. Potass. hydroxide 0·5% may be used during the last few days of the clearing.

4. Wash twenty-four hours in running tap water.

5. Stain one half to six hours, according to the size of the specimen, in Solution A.

6. Wash for thirty minutes in running tap water.

7. Decolorize seven to fourteen days in Solution B.

8. Mount in a glass frame and dehydrate by passing slowly through alcohol-glycerin-water mixtures beginning with the proportions 1 : 2 : 7 and then in succession 2 : 2 : 6, 3 : 3 : 4, 4 : 4 : 2; and finally equal parts of alcohol and glycerin only.

9. Seal in the usual glycerin-alcohol mixture.

For nervous tissues (Benda's method)

Solutions required:

A. Nitric acid, conc. .. .. 1 volume
Distilled water .. .. .. 10 volumes

B. Potass. dichromate 2%

C. Chromic acid 1%

D. Iron alum 4%

E. Alizarin Red, S, saturated in absolute alcohol .. .. .. 1 ml.
Distilled water .. .. .. 90 ml.

F. Toluidine Blue 0·1% aqueous

Technique:

1. Material is fixed in 90–95% alcohol for at least two days.

2. Pieces, which must not be thicker than 0·5 cm., are immersed in Solution A for twenty-four hours.

3. Transfer to Solution B for twenty-four hours.

4. Transfer to Solution C for forty-eight hours; then wash in water for twenty-four hours.

5. Dehydrate in the usual manner.

6. Clear in beechwood creosote for twenty-four hours; then in benzol for twenty-four hours.

7. Embed in a saturated solution of paraffin wax in benzol at room temperature; then successively in saturated solutions of paraffin wax in benzol at 38° C., 42° C. and 45° C. so that pure paraffin wax only is used for the final embedding.

8. Mount sections on slides: bring down to distilled water, mordant sections on slides with Solution D for twenty-four hours, then wash thoroughly in water.

9. Stain for two hours with Solution E; then rinse in tap water.

10. Flood slides with Solution F and warm gently until vapour is given off; or stain at room temperature for 24 hours.

11. Rinse in 1% acetic acid; then dry by blotting carefully.

12. Pass through absolute alcohol; then differentiate for about ten minutes in beechwood creosote; dry by blotting carefully, wash with xylol, and mount.

Vital staining of nervous tissue in small vertebrates

Solution required:

Alizarin Red, S, 2% aqueous

Technique:

1. Paraffin sections are brought down to distilled water by the usual method.

2. Stain twenty-four hours in 2% aqueous Alizarin Red, S.

3. Differentiate thirty to sixty seconds in distilled water to which has been added three drops 1% calcium acetate per 10 ml.

4. Dehydrate: clear and mount.

Note: This is a general stain which also demonstrates Nissl bodies as well as other details.

ALUM CARMINE - ANILINE BLUE - ORANGE G.

For demonstrating the various components of the Hypophysis

Solutions required

A. Cresofuchsin.

B. Alum carmine (Mayer).

C. Orange G .. .. .. 2 gm.
Phosphomolybdic acid .. .. 1 gm.
Distilled water .. .. .. 100 ml.

D. Phosphomolybdic acid 5% aqueous.

E. Aniline Blue 0·2% aqueous.

Technique:

1. Fix in 10% formalin; harden and dehydrate in graded alcohols; clear in chloroform: embed in paraffin wax.

2. Sections are brought down to 70% alcohol and stained for two to twenty-four hours in Solution A.

3. Wash quickly with distilled water; then stain with Solution B for three hours, afterwards washing with distilled water.

4. Differentiate and stain the acidophil cells for five minutes with Solution C; then rinse in distilled water.

5. Immerse in Solution D for two minutes; then blot dry.

6. Stain ten to twenty minutes with Solution E.

7. Rinse in distilled water; differentiate with 75% alcohol until no more stain comes out; then dehydrate; clear in xylol and mount.

Results:

Chief cells, blue to grey. Pregnancy cells, blue with small bright yellow granules; basophiles with coarse reddish blue granules. Epithelium of the pars intermedia and pars tuberalis, variable. Collagen fibre, intense blue. Glia fibres, blue-grey; axons, occasionally black.

AMMONIACAL SILVER CARBONATE

For vascular reticulum, tumour cells, connective tissues around tumour, in abnormal brain tissue

Solutions required:

A. Pyridin, pure .. .. .. 2 volumes
Glycerin, pure .. .. .. 1 volume

B. *Ammoniacal Silver Carbonate.*

Ammonia solution is added drop by drop to 10 ml. silver nitrate 10·2% until the precipitate formed is almost redissolved, leaving a slightly opalescent solution to which is then added 10 ml. sodium carbonate 3·1% solution and sufficient distilled water to make the volume up to 100 ml.

C. *Reducing solution:*

Sodium carbonate anhydrous .. 1 gm.
Formalin .. .. 1 ml.
Distilled water .. .. .. 103 ml.

D. Brown gold chloride 0·2% aqueous.

E. *Intensifying solution:*

Oxalic acid 2% aqueous .. 100 ml.
Formalin .. .. 1 ml.

F. Sodium hyposulphite 10% aqueous.

N.B.—All the above solutions must be kept in dark bottles.

Technique:

The material is fixed in 10% formalin or in Bouin and embedded in paraffin wax.

1. Bring sections down to distilled water and immerse in Solution A for twenty-four hours.

2. Wash with 95% alcohol, then with distilled water.

3. Immerse in Solution B for two and a half hours at 40° C.

4. Wash with distilled water; then reduce in Solution C for five minutes, afterwards washing in tap water.

5. Tone for five minutes in Solution D at 30° C.; then wash in tap water.

6. Intensify by immersing in Solution E for five minutes; then rinse in tap water.

N.B.—The above stages must be carried out in the darkroom.

7. Fix in Solution F. (*Note:* Fixation should be completed in fifteen to twenty minutes.)

8. Wash in tap water; dehydrate; clear and mount.

Results:

Tumour cells: reddish to greyish violet. Vascular reticulum: black.

Important.—The tissues must not be allowed to come into contact with mercuric chloride, as even a trace will ruin the preparation.

ANILINE BLUE - ACID FUCHSIN

For elementary bodies in animal sections

Solutions required:

A.	Picric acid, saturated alcoholic,	10 ml.
	Formalin	25 ml.
	Absolute alcohol	65 ml.
	Glacial acetic acid	5 ml.
B.	Aniline blue, water soluble	1 gm.
	Distilled water	65 ml.
	Methyl alcohol, pure	35 ml.
	Glycerin, pure	5 ml.
	Oxalic acid 3% aqueous	2 ml.
C.	Acid fuchsin 1% aqueous	100 ml.
	Oxalic acid 3%	2 ml.

Technique:

1. Pieces of tissue are fixed for twenty-four hours in Solution A; washed, dehydrated, cleared and embedded in paraffin wax as usual.

2. Sections, not thicker than 5μ, are fixed to slides, de-waxed and taken through descending grades of alcohol down to water as usual.

3. Stain for one half to one hour in the aniline blue (Solution B) in a stoppered staining jar.

4. Rinse well with distilled water.

5. Drain and blot carefully to remove excess water.

6. Rinse in absolute alcohol.

7. Stain for twenty minutes in the acid fuchsin (Solution C).

8. Pour off; drain and blot carefully to remove excess liquid.

9. Dehydrate with absolute alcohol; clear in xylol and mount in cristalite or in Canada balsam.

Results:

Elementary bodies in cells associated with the following viruses are stained scarlet: borna, zoster, rabies and pseudo rabies.

ANILINE CRYSTAL VIOLET - GRAM'S IODINE

For epithelial fibres

Solutions required:

A. Aniline crystal violet.

B. Gram's iodine.

C. Aniline xylol.

Technique:

1. Material should be fixed in absolute alcohol and embedded in paraffin wax.

2. Sections, not more than 5μ thick, are fixed to slides and brought down to distilled water in the usual manner.

3. Stain for ten to fifteen minutes in aniline crystal violet.

4. Wash well in running water.

5. Stain with Gram's iodine for ten to thirty seconds.

6. Wash in water; drain; then blot carefully but thoroughly to remove water.

7. Differentiate with aniline xylol, controlling at frequent intervals by examination under the microscope.

8. Wash well with xylol; mount in balsam or D.P.X.

Results:

Epithelial fibres are stained blue.

ANILINE CRYSTAL VIOLET - LITHIUM CARMINE - IODINE

For fibrin and for Gram-positive organisms in animal tissues

Solutions required:

A. Lithium carmine

B. Crystal violet .. .. .. 1 gm.
Aniline oil.. .. .. .. 3 ml.
Absolute alcohol .. .. 10 ml.
Dissolve and filter.

C. Crystal violet 2% aqueous.

D. Solution B .. .. .. 3 ml.
Solution C .. .. .. 27 ml.
This mixture should be prepared immediately before use.

E. Gram's iodine.

F. Aniline oil.. .. .. .. 1 volume
Xylol .. .. 1 volume

Technique:

1. Fix material in absolute alcohol, Carnoy or alcohol-formalin and embed in paraffin wax.

2. Fix sections to slides; de-wax and pass through descending grades of alcohol down to distilled water in the usual way.

3. Stain in the lithium carmine solution for two to five minutes.

4. Wash thoroughly in distilled water.

5. Immerse in Solution D for five to ten minutes.

6. Rinse in distilled water; drain well and blot carefully.

7. Cover with Gram's iodine solution and allow the stain to act for five to ten minutes.

8. Pour off the excess iodine solution and blot carefully with filter paper.

9. Differentiate with the aniline xylol solution until no more purple coloration comes out.

10. Drain, and blot carefully.

11. Rinse with several changes of xylol.

12. Mount in balsam or in cristalite.

Results:

Fibrin and Gram-positive organisms are blue to blue-black while nuclei are red.

ANILINE BLUE - ORANGE G (Mallory)

For collagenous and reticulin fibrils; cartilage, bone, amyloid, nuclei; fibroglia and elastin fibres

Solutions required:

A. Acid fuchsin ½–1% aqueous.

B. Aniline Blue - Orange G.

Technique:

Tissues are fixed in Zenker and embedded in paraffin wax, Celloidin or L.V.N.

1. Mount sections on slides and bring down to 90% alcohol; then treat with iodine in the usual way to remove mercuric deposits.

2. Bring down to distilled water and stain for one to ten minutes in Solution A; then without washing:

3. Stain for twenty minutes to one hour or longer in Aniline Blue - Orange G; then remove excess stain with several changes of 95% alcohol.

4. Dehydrate with absolute alcohol; clear in xylol and mount in Cristalite.

Note: If Celloidin or L.V.N. sections are used the staining time may be shortened and 95% alcohol should be used for decolorizing and dehydration; terpineol for clearing.

Results:

Collagenous fibril, intense blue. Ground substances of cartilage, bone, mucus, amyloid: varying shades of blue. Nuclei, myoglia, neuroglia fibrils, axis cylinders, fibrin, nucleoli: red. Blood corpuscles and myelin: yellow. Elastic fibrils: pale pink or pale yellow, or unstained; fibriloglia: red or unstained.

Note: By omitting the acid fuchsin the collagenous fibres are more sharply defined.

AZAN STAIN

(Heidenhain)

Solutions required:

A.
Azocarmine B	0·5 gm.
Distilled water	100 ml.
Glacial acetic acid	1 ml.

Dissolve by warming; cool and filter.

B.
96% alcohol	100 ml.
Aniline oil	0·1 ml.

C.
95% alcohol	99 ml.
Glacial acetic acid	1 ml.

D.
Phosphotungstic acid	5 gm.
Distilled water	75 ml.
Methyl alcohol	25 ml.

E.
Aniline Blue, water soluble	0·5 gm.
Orange G	2 gm.
Glacial acetic acid	8 ml.
Distilled water	100 ml.

Dissolve by warming; cool and filter.

For staining, dilute 1 volume of this solution with 3 volumes of distilled water.

Technique:

1. Zenker-, Bouin- or Carnoy-fixed tissues are stained from forty-five to sixty minutes at 55° C. in Solution A; then at room temperature for five to ten minutes.

2. Wash in distilled water; then differentiate in Solution B until cytoplasm is pale pink, and nuclei are red and clear.

3. Rinse for one half to one minute in Solution C.

4. Transfer to Solution D for about one to three hours or until the connective tissue is completely decolorized; then wash quickly in distilled water.

5. Stain for one to two hours in the diluted Solution E, examining at ten- or fifteen-minute intervals to prevent over-staining.

6. Wash quickly in distilled water; then differentiate in 95% alcohol followed by absolute alcohol.

7. Clear and mount.

Results:

Collagen stained deep blue; reticulum, deep blue; chromatin, red; muscle tissue, reddish to orange; erythrocytes, red; neuroglia, reddish; mucin, blue.

AZO CARMINE - MALLORY STAIN

For Islets of Langerhans

Solution required:

A. Azocarmine, B, aqueous 0·1% .. 100 ml.
Boil for about 5 minutes; then cool and add 2 ml. glacial acetic acid. Then warm to 60 °C. and filter at that temperature.

B. 90% alcohol 99 ml. + 1 ml. Aniline Oil.

C. Iron alum 5% aqueous.

Aniline Blue - Orange G (Mallory):

D.	Aniline blue, aqueous	0·5 gm.
	Orange G.	2 gm.
	Distilled water	100 ml.
E.	Solution D	1 volume
	Distilled water	2 to 3 volumes

Technique:

1. Fix thin slices of pancreas in Bouin for eight to ten hours.

2. Wash in distilled water, dehydrate, clear and embed in paraffin wax.

3. Cut section 4μ in thickness.

4. Fix section to slides; dewax and take down through the usual grades of alcohol to distilled water.

5. Stain in solution A for about forty-five to sixty minutes at 56° C.

6. Rinse quickly in distilled water and blot very carefully.

7. Destain in solution B until acinous tissue is almost colourless and B cells show red against pink background of A cells.

8. Rinse briefly with distilled water and treat with 5% iron alum solution for 5 minutes or more.

9. Rinse again and stain two to twenty minutes in solution E until the collagenic tissue appears deep blue under the microscope.

10. Rinse and blot carefully.

11. Differentiate and dehydrate in absolute alcohol.

12. Clear in xylol and mount.

Results:

Cytoplasm of A cells: rich yellow orange; of B cells: bright red; and of D cells: sky blue.

Note: **It is stated that it can be demonstrated that there is no gradation between A and B cells by first staining with Neutral Gentian (Bensley) decolorizing, then restaining by the above technique.**

Reference:

Gomori, G. (1939), *Anat. Rec.*, **74**, 439–459.
Cowdry, E. V.: *Laboratory Technique*, 3rd ed., p 167.

AZOCARMINE - HAEMATOXYLIN - ACID GREEN - ORANGE G

For differential cell analysis of the rat anterior hypophysis

Solutions:

A. *Zenker - Formol*

Potassium dichromate	25 gm.
Mercuric chloride	50 gm.
Ringer's solution (i.e. 0·9% saline) ..	1 litre.

Add 1 ml. neutral formaldehyde 40% solution per 10 ml. of the above solution immediately before using.

B. Lugol's iodine

C. Sodium thiosulphate 0·5% aqueous

D. Delafield Haematoxylin

E. Alcohol 95%	999 ml.
Aniline oil	1 ml.
F. Azocarmine G or B 1% aqueous ..	100 ml.
Glacial acetic acid	4 ml.
G. Glacial acetic acid	10 ml.
Alcohol 90%	90 ml.

H. Phosphotungstic acid 5%

I. Acid green L extra	0·1 gm.
Orange G	0·5 gm.
Clove oil	100 ml.

N.B. Use fresh stain for each batch of about twenty slides.

Technique:

1. Fix in solution A for six to twelve hours.

2. Wash six to twelve hours in running water.

3. Dehydrate by immersing for thirty minutes in each of the following: 30%, 50%, 70%, 80% and 95% alcohol.

4. Immerse in absolute alcohol, two changes, for one hour in each.

5. Immerse in a mixture consisting of equal parts of absolute alcohol and cedarwood oil for one hour.

6. Cedarwood oil, one to sixteen hours.

7. Xylol, for fifteen minutes.

8. Infiltrate in paraffin wax 56–58° C. (four changes before finally embedding).

9. Fix sections, 4μ in thickness, to slides and remove paraffin wax by immersing in two changes of xylol for three minutes each.

10. Immerse for three minutes in each of two changes of absolute alcohol.

11. In 95% alcohol for three minutes.

12. Distilled water for three minutes.

13. Lugol's Iodine for three minutes.

14. Sodium thiosulphate solution for three minutes when the sections should have been restored to their natural colour.

15. Stain in Delafield Haematoxylin for thirty seconds.

16. Wash in tap water for three minutes.

17. Immerse in distilled water for three minutes.

18. Immerse in 80% alcohol for three minutes.

19. Aniline alcohol (Solution E) for fifteen minutes.

20. Stain in Azocarmine for forty-five minutes.

21. Rinse in distilled water.

22. Differentiate in aniline alcohol for two to three minutes.

23. Wash in acid alcohol (solution G) for thirty to sixty seconds.

24. Immerse in phosphotungstic acid solution for one hour.

25. Dehydrate by passing through 70%, 95% and absolute alcohols (two minutes in each).

26. Counterstain in acid green - orange G solution for five minutes.

27. Clear in xylol for one minute.

28. Immerse for half an hour in each of two changes of xylol, to remove completely all traces of clove oil which would otherwise cause further decolorisation.

29. Mount in D.P.X. or Clearmount.

Results:

Alpha granules, purplish red, Beta cell granules, light green. Nuclear membranes are sharply defined and mitochondria are

orange red. Erythrocytes, brilliant orange. Golgi apparatus shows as negative image in both alpha and beta cells. Chromophobes show little or no cytoplasm, which is colourless to pale green.

Note: A method of counting the cells is given in the original paper.

Reference: Briseno-Castrejon, B. and Finerty, J. C. (1949), *Stain Tech.*, **24**, 103–7.

BAUER - FEULGEN STAIN

For Glycogen, etc.

Solutions required:

A. Chromic acid 4% aqueous

B. Feulgen's fuchsin

C. Sodium metabisulphite 10·4% aqueous .. .. 5 ml.
Tap water .. .. 95 ml.

Technique:

1. Fix fresh material immediately in alcohol and embed in paraffin wax.

2. Dewax sections; pass through the usual descending grades of alcohol to distilled water.

3. Immerse in the chromic acid solution for one hour.

4. Wash in running water for five to ten minutes.

5. Immerse in the Feulgen fuchsin for ten to twenty minutes.

6. Agitate the slides gently for about two minutes in each of three changes of solution C.

7. Wash in running water for ten minutes.

8. Stain nuclei, if desired, in haemalum, for two to five minutes.

9. Dehydrate as usual; clear in xylol and mount.

Results:

Glycogen, intense reddish-violet. Nuclei, pale mauve to navy blue.

BIEBRICH SCARLET - ETHYL VIOLET - HAEMATOXYLIN

(Cambel and Sgouris modification of Bowie's Stain) for pepsinogen granules of the body chief cells in the gastric glands

Solutions required:

A. Delafield Haematoxylin, aqueous

B. Ethyl Violet - Biebrich Scarlet .. 0.5 gm.
20 % Ethyl alcohol .. .. .. 50 ml.

C. Solution B. .. .. 0·5 gm.
Absolute alcohol .. .. .. 20 ml.

Distilled water .. .. 80 ml.

N.B.—This solution must be freshly prepared each time it is required for use.

D. Clove Oil .. .. 1 volume
Toluol .. .. 1 volume

Technique:

1. Fix in Regaud's fluid for five days in a dark phial placed in a larger amber bottle which should be wrapped round with a thick cloth and kept in a dark room. Change the Fixative daily.

2. Dehydrate with normal propyl alcohol (*see* page 38).

3. Transfer to paraffin wax which should be changed three times before the block is finally cast.

4. Cut sections 5 to 6μ in thickness and fix to slides as usual.

5. Remove wax with two changes of xylol.

6. Pass through absolute alcohol and the usual ascending grades of alcohols down to distilled water.

7. Stain in the Haematoxylin solution for 1 minute.

8. Wash and blue in tap water.

9. Remove excess water by draining and blotting very carefully.

10. Stain with solution B for ten to fifteen minutes, or longer (up to twenty-four hours).

11. Rinse briefly in distilled water, then drain and carefully remove excess water by blotting.

12. Differentiate in solution D (clove-toluol), controlling under the microscope, for about ten to fifteen minutes.

13. Rinse with two changes of toluol.

14. Mount in permount-toluene or in Clearmount or Cristalite.

Results:

Zymogen granules, dark violet. Parietal cells, scarlet, and nuclei, blue. The parietal cells are distinctly contrasted from the pepsinogen cells.

Reference: Cambel, P. and Sgouris, J. (1951), *Stain Tech.*, **26**, 243–6.

BISMARK BROWN - METHYL GREEN

For mucin, cartilage, and goblet cells in embryonic tissue, trachea and intestine

Solutions required:

A. Bismark brown 1% aqueous.

B. Methyl green 0·5% aqueous.

Technique:

Tissues are fixed in Bouin or Zenker and embedded in paraffin wax.

1. Sections are brought down to distilled water; then stained five to ten minutes in Solution A.

2. Wash with 95% alcohol.

3. Stain with Solution B until the preparation appears dark green to the naked eye.

4. Dehydrate with 95% and absolute alcohol; then clear in xylol, and mount.

Results:

Cartilage: dark brown. Mucin: light brown. Nuclei of all cells: green.

BIONDI - EHRLICH - HEIDENHAIN STAIN

For chromatin, nucleoli, mucin, etc.

Solution required:

Biondi - Ehrlich - Heidenhain	0·9 gm.
Distilled water	100 ml.

Dissolve by warming and stirring in a beaker. When cool add:

Chloroform	0·25 ml.

Technique:

1. Fix tissues in saturated aqueous mercuric chloride and embed in paraffin wax in the usual manner.

2. Fix sections to slides; de-wax with xylol and pass through absolute alcohol followed by 90% and 70% alcohol.

3. Treat for the removal of mercuric precipitate by the standard technique (*see* page 28).

4. Immerse in the staining solution from six to twenty-four hours.

5. Rinse directly with 95% alcohol.

6. Dehydrate with absolute alcohol.

7. Clear in xylol and mount.

Results:

Chromatin is stained bluish green, while nucleoli are red; mucin is stained green; erythrocytes, orange. Cytoplasm and connective tissue elements are in varying shades of red.

BEST'S CARMINE

For glycogen

Note: This method has the advantage over the Lugol's iodine technique in that fading does not occur so readily, and better staining of glycogen is obtained. The disadvantages are that the stain is less specific than in the iodine method, and the solution

deteriorates after a few weeks. If negative results are obtained the stain should be checked by staining a section known to contain glycogen.

Solutions required:

A. Ehrlich haematoxylin.

B. Best's carmine stock solution .. 10 ml.
Methyl alcohol, pure .. .. 15 ml.
Strong ammonia solution .. 10 ml.

Note: This solution should be prepared immediately before it is required for use.

C. Celloidin 1% in equal volumes of absolute alcohol and ether.

D. Absolute (ethyl) alcohol .. .. 80 ml.
Absolute (methyl) alcohol .. 40 ml.
Distilled water .. .. .. 100 ml.

Technique:

1. Tissues are fixed in Bouin Fluid and embedded in Celloidin or in paraffin wax. If Celloidin sections are employed proceed as from stage 5 (below). If paraffin sections are used the procedure is as follows:

2. Float sections on the slide with 70% alcohol; flatten out; then remove excess alcohol with filter paper and blot carefully but thoroughly.

3. Remove paraffin wax with xylol in the usual manner.

4. Wash with absolute alcohol as usual.

5. Transfer the slide to a stoppered staining jar containing 1% Celloidin (Solution C, above), for fifteen minutes.

6. Transfer to a stoppered jar containing 70% alcohol, after rapidly wiping off the Celloidin from the back of the slides. This operation must be carried out quickly so that the Celloidin is not allowed to dry. Leave in the alcohol from ten to fifteen minutes.

7. Transfer to Ehrlich haematoxylin and allow the stain to act from two to ten minutes, differentiating if necessary with acid alcohol, controlling under the microscope.

8. Rinse in water, and without " blueing " in tap water, transfer to Best's carmine solution (formula as above) and allow the stain to act for five to ten minutes.

9. Differentiate in Solution D (above) from one to five minutes until the stain ceases to come away from the section.

10. Transfer to a mixture consisting of equal volumes of ether and absolute alcohol to dissolve out Celloidin and to dehydrate.

11. Clear with xylol and mount.

Results:

Glycogen is stained as brilliant red granules, while nuclei are blue.

BENZIDINE

For brain capillaries

Solution required:

A. Benzidine base, pure .. .. 1 gm.
Acetic acid 2·5% aqueous .. 200 ml.

B. Sodium nitroprusside 1% aqueous

C. Solution A .. .. .. 20 ml.
Solution B .. .. .. 10 ml.
Distilled water .. .. .. 70 ml.
Mix well and filter.

N.B.: This mixture should be prepared immediately before use.

D. Distilled water .. .. .. 100 ml.
Hydrogen peroxide 20 vols. .. 1·5 ml.

Technique:

1. Tissue should be fixed for one to three weeks in 10% formalin and frozen sections, 200 to 300μ should be employed.

2. Immerse in several changes of distilled water for a total period of two hours.

3. Immerse in Solution C for half an hour at 37° C. agitating at frequent intervals.

4. Wash in several changes of distilled water.

5. Immerse in Solution D for half an hour at 37° C. agitating frequently.

6. Wash in distilled water.

7. Dehydrate by passing through ascending strengths of alcohol, beginning with 70%, in the usual manner.

8. Clear in xylol and mount.

Results:

Blood vessels are stained black against a background which is almost colourless.

BASIC FUCHSIN - METHYLENE BLUE

For demonstrating Negri bodies in sections

Solution required:

Stock solutions:

A.	Basic fuchsin	1·5 gm.
	Methylene Blue	1 gm.
	Glycerine	100 ml.
	Methyl alcohol, pure	100 ml.
B.	Potassium hydroxide aqueous 0·25%	1 ml.
	Distilled water	99 ml.

Staining solution:

C.	Solution A	40 ml.
	Solution B	1 ml.

N.B.: This solution must be freshly prepared immediately before use.

Technique:

1. Pieces of fresh tissue, from the hippocampus major and cerebellum, not more than 3 mm. in thickness are fixed in Zenker, washed, dehydrated, cleared, and embedded in paraffin wax.

2. Fix sections to slides; de-wax with xylol.

3. Pass through descending grades of alcohol and treat for the removal of mercuric precipitate left by the fixative by the standard technique.

4. Place slides, section facing upwards, over the corner of a tripod.

5. Flood slide with the staining solution (Solution C, above) and heat gently with a small bunsen flame for five minutes with steam rising, taking care that the preparation does not catch fire.

6. Allow the slide to cool for a few seconds; then wash quickly in water.

7. Decolorize and differentiate in 90% alcohol until the sections assume a faint violet colour.

8. Rinse quickly in 95% alcohol.

9. Dehydrate rapidly with absolute alcohol.

10. Clear in xylol and mount.

Results:

Negri bodies are stained deep red, while the granular inclusions are dark blue. Nucleoli are blue-black, while cytoplasm is bluish violet, and erythrocytes appear copper coloured.

BASIC FUCHSIN - GENTIAN VIOLET - IODINE

For bacteria in sections

Solutions required:

A. Basic fuchsin .. .. .. 0·75 gm.
Alcohol absolute .. .. 30 ml.
Phenol crystals .. .. .. 1 gm.
Distilled water .. .. .. 100 ml.

B. Picric acid, saturated, aqueous.

C. Aniline gentian violet.

D. Gram's iodine.

E. Aniline oil.. .. .. 1 volume
Xylol .. .. 1 volume

Technique:

1. Pieces of tissue should be fixed in Zenker's fluid, washed in running water, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Fix thin sections to slides, de-wax, and treat for the removal of mercuric precipitate by the standard technique.

3. Stain in the fuchsin solution for ten to thirty minutes.

4. Differentiate in formalin until the bright red colour is reduced to pink.

5. Wash in distilled water.

6. Counterstain in the picric acid solution for a few minutes until the preparation assumes a purplish yellow colour.

7. Wash in distilled water.

8. Differentiate in 95% alcohol until the sections are red again, and yellow and red coloration begins to come out.

9. Rinse in distilled water.

10. Stain in aniline gentian violet for five to ten minutes.

11. Rinse in distilled water.

12. Stain in Gram's iodine solution for one minute.

13. Drain and blot dry.

14. Differentiate with aniline-xylol until colour ceases to come out of the sections.

15. Clear with two changes of xylol and mount.

Results:

Gram-positive organisms are stained blue, while Gram-negative are red. Tissue, red and blue; fibrin, deep blue.

CARBOL ANILINE FUCHSIN

For Negri bodies

Solutions required:

A. Basic fuchsin .. .. .. 0·5 gm.
Distilled water .. .. .. 80 ml.
Absolute alcohol .. .. .. 20 ml.
Aniline oil.. .. .. .. 1 ml.
Phenol .. .. 1 gm.

B. Methylene Blue (Loeffler).

Technique:

1. Fix tissues in Zenker's Fluid for twenty-four hours; wash in running water for two or three hours; dehydrate; clear; embed in paraffin wax in the usual manner.

2. Sections, 4 to 5μ in thickness, are stained from ten to thirty minutes in Solution A; then washed with distilled water.

3. Stain with Methylene Blue (Loeffler) for fifteen to sixty seconds; then wash with water.

4. Dehydrate and differentiate for a few seconds in absolute alcohol; then clear in xylol and mount.

Results:

Negri bodies are stained crimson against a blue background.

CARBOL FUCHSIN (Ziehl Neelsen)

For Nissl Bodies

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen) .. 10 ml.
Acetic acid 0·5% .. .. 10 ml.

B. Acetic acid 1% .. .. .. 10 ml.
Formalin .. .. 0·1 ml.

Technique:

1. Small pieces of tissue are fixed in 10% formalin or in 95% alcohol or in physiological saline for at least twenty-four hours, and afterwards washed, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Stain sections for three to four minutes with the carbol fuchsin (Solution A).

3. Wash quickly in distilled water; then de-stain in Solution B.

4. Wash in distilled water; then dehydrate.

5. Clear in xylol; then mount.

Results:

Nissl bodies and nucleoli are stained dark red; remainder unstained.

CARBOL FUCHSIN - BORREL BLUE

For Leprosy and for T.B.

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Sulphuric acid 5%.

C. Hydrochloric acid .. .. 1 ml.
Alcohol 70% .. .. .. 99 ml.

D. Borrel's Blue .. .. .. 5 ml.
Distilled water .. .. .. 20 ml.

Technique:

1. Material should be fixed in saturated aqueous solution of mercuric chloride and embedded in paraffin wax.

2. Fix sections to slides and treat them for the removal of mercuric precipitate by the standard method (*see* page 28).

3. Immerse in carbol fuchsin in a staining jar for thirty minutes to an hour in the incubator at 37° C.

4. Decolorize in Solution C.

5. Decolorize in 70% alcohol (neutral) for two or three minutes until the sections appear faintly pink to the naked eye.

6. Counterstain in Borrel's Blue (diluted as above: Solution D) for one or two minutes.

7. Rinse in distilled water; drain and carefully blot away excess water.

8. Dehydrate and differentiate the Borrel Blue, controlling by examination under the microscope.

9. Clear in xylol and mount.

Results:

T.B. or leprosy, bright red; other bacteria, blue; cells and cell debris, varying shades of blue; cell nuclei, blue.

N.B.: For demonstrating leprosy, differentiation of the carbol fuchsin (stages 4 and 5) must be very carefully carried out, as this organism is more easily completely decolorized than T.B.

CARBOL FUCHSIN - HAEMATOXYLIN

For tubercle bacilli in mammalian tissue

Solutions required:

A. *Alum Haematoxylin:*

Potash alum	20 gm.
Haematoxylin	1 gm.
Thymol	1 gm.
Distilled water	400 ml.

B. Carbol fuchsin (Ziehl Neelsen) 1 volume
Distilled water .. .. .. 3 volumes

Technique:

Tissues are fixed in Zenker or Flemming and embedded in paraffin wax, L.V.N. or Celloidin.

1. Paraffin sections are brought down to distilled water; then stained one to five minutes in Solution A.

2. Differentiate if necessary with acid alcohol, controlling under the microscope, till nuclear detail is sharp and clearly defined; wash thoroughly in water.

3. Stain with Solution B for five minutes, heating till steam rises; or stain at room temperature overnight.

4. Decolorize for twenty seconds in acid alcohol; then wash thoroughly in water to which two or three drops of ammonia have been added to remove the acid.

5. Differentiate in 95% alcohol.

6. Dehydrate; clear and mount.

If Celloidin or L.V.N. sections are employed, clear in terpineol or origanum oil; blot carefully on slide and mount.

Results:

Tubercle bacilli, bright red. Nuclei, blue.

CARBOL FUCHSIN - HAEMATOXYLIN - PICRO ACID FUCHSIN

For M. leprae in sections

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Hydrochloric acid, conc. .. 3 ml.
Absolute alcohol .. .. 97 ml.

C. Potassium permanganate 1% aqueous.

D. Oxalic acid 2% aqueous.

E. Haematoxylin (Ehrlich).

F. Picric acid, saturated, aqueous .. 50 ml.
Acid fuchsin, aqueous 1% .. 10 ml.
Distilled water .. .. 40 ml.

Technique:

1. Pieces of tissue are fixed for three to seven days in a mixture consisting of equal volumes of 10% formalin and absolute alcohol, and paraffin sections are employed.

2. Stain sections in the carbol fuchsin solution in a stoppered staining jar for three or four days.

3. Immerse in 10% formalin, of a slightly acid reaction, for five minutes.

4. Immerse in the acid alcohol for five minutes.

5. Flood the preparation with potassium permanganate and allow the reagent to act until the sections turn brown (this usually takes from two to five minutes).

6. Immerse in the oxalic acid for one minute.

7. Stain with Ehrlich haematoxylin solution for two minutes; then blue in tap water or in saturated lithium carbonate solution aqueous.

8. Stain in picro-acid fuchsin for two to five minutes; then without washing:

9. Dehydrate, clear and mount.

Results:

M. leprae, dark blue. Connective tissue fibres, red. Muscle, yellow. Nuclei, brown.

CARBOL FUCHSIN - IODINE - HAEMATOXYLIN ORANGE G

For demonstrating leprosy organisms together with neurokeratin of the myelin sheath

Solutions required:

A. Lugol's iodine.

B. Carbol fuchsin (Ziehl Neelsen).

C. Absolute alcohol .. .. .. 35 ml.
Distilled water .. .. .. 65 ml.
Hydrochloric acid concentrated.. 0·5 ml.

D. Ehrlich's haematoxylin.

E. Strong ammonia solution (sp. gr. 0·880) .. .. 1 ml.
Distilled water .. .. .. 99 ml.

F. Orange G. aqueous 1%.

Technique:

1. Pieces of tissue are fixed in Zenker; washed; then transferred to a mixture of Lugol's iodine and 80% alcohol (equal volumes of each) for six to twenty-four hours.

2. Transfer to 80% alcohol for twelve to twenty-four hours.

3. Immerse in 95% alcohol for two to six hours.

4. Transfer to a mixture consisting of equal volumes of absolute alcohol and xylol, for half an hour.

5. Immerse in xylol for half an hour.

6. Immerse in two changes of paraffin wax before casting the block and finally sectioning.

7. Fix sections to slides and remove wax with xylol.

8. Pass through absolute, 90% and 70% alcohol.

9. Stain for half an hour in carbol fuchsin (Solution B).

10. Rinse in distilled water.

11. Partially differentiate with the acid alcohol (Solution C, above).

12. Rinse well with distilled water.

13. Stain for one to two minutes with Ehrlich haematoxylin solution.

14. Differentiate in the acid alcohol (Solution C).

15. Rinse well with distilled water.

16. Immerse in the ammonia solution (Solution E above) for a few seconds.

17. Rinse well with distilled water.

18. Stain with the Orange G solution for two to three minutes.

19. Dehydrate rapidly with two changes of acetone.

20. Clear in xylol and mount.

Results:

Leprosy organisms and neurokeratin are stained red, while nuclei are blue and cytoplasm is yellow.

CARBOL FUCHSIN - METHYL GREEN

For demonstrating hyaline substance

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen) .. 5 ml.
Distilled water .. .. .. 45 ml.

B. Methyl Green 1% in 5% acetic acid.

Technique:

1. Material which has been fixed in any of the standard fixatives is embedded in paraffin wax.

2. Fix sections to slides: then bring down to distilled water as usual.

3. Stain in Solution A for fifteen to forty-five minutes.

4. Wash with distilled water; drain off excess; then blot carefully.

5. Dehydrate rapidly with absolute alcohol.

6. Differentiate and counterstain in Solution B for two or three minutes.

7. Wash quickly with absolute alcohol.

8. Clear in xylol and mount.

Results:

Hyaline substance is stained bright red while nuclei are light green.

CARBOL THIONIN - PICRIC ACID (Schmorl)

For demonstrating bone canaliculi

Solutions required:

A. *Decalcifying solution:*
Formalin 10% .. .. .. 100 ml.
Nitric acid, conc. .. .. 15 ml.

B. Carbol thionin (Nicolle).

C. Picric acid 1% aqueous.

Technique:

1. Formalin-fixed specimens are placed in a large volume of Solution A, which is changed once or twice a day. The time required for complete decalcification will depend, of course, on the thickness and the nature of the specimen. The bones of young animals usually take from twenty-four to forty-eight hours, but in other cases as long as a week may be necessary. Decalcification is complete when the bone has become flexible and can easily be punctured with a needle.

2. Make Celloidin or frozen sections.

3. Rinse in water for ten minutes.

4. Stain with Solution B for 10 minutes; then rinse in distilled water.

5. Immerse in Solution C for half to one minute; then wash in water.

6. Differentiate with 70% alcohol for five to ten minutes until the stain ceases to come out of the sections.

7. Dehydrate with 96% alcohol; clear in origanum oil; then mount in balsam.

Results:

Ground substance, yellow to brown; bone canaliculi, dark brown to black; cells, red; ground substance of cartilage, brilliant purple.

Note: Carbol thionin (Nicolle) deteriorates after a few weeks and this stain is therefore best when freshly prepared.

CARMINE - METHYLENE BLUE (Schultz-Schmitz Stain)

For demonstrating sodium urate in animal tissue

Solutions required:

A. Distilled water .. .. .. 64 ml.
Lithium carbonate .. .. 0·5 gm.
Carmine .. .. 1 gm.
Ammonium chloride .. .. 2 gm.
Boil for a few minutes; allow to cool; then make up to the original volume and add:
Strong ammonia solution .. 6 ml.
Filter before use.

B. Solution A .. .. .. 15 ml.
Pure methyl alcohol .. .. 12·5 ml.
Strong ammonia solution (sp. gr. 0·880) .. .. 2 ml.
Distilled water .. .. .. 5·5 ml.

C. Methylene Blue 1% in absolute alcohol.

D. Picric acid, saturated aqueous .. 27 ml.
Sodium sulphate saturated aqueous .. .. .. 3 ml.

Technique:

1. Fix thin slices of the material in absolute alcohol.

2. Immerse for one-and-a-half to two hours in each of three changes of acetone.

3. Transfer to a mixture of equal volumes of acetone and benzol for half an hour.

4. Immerse in pure benzol for one half to one hour; then embed in paraffin wax.

5. Fix sections to slides and de-wax with xylol.

6. Pass through absolute, followed by 90% alcohol.

7. Immerse in Solution B in a grooved staining jar for five minutes, rocking gently, but continuously, during the period of staining.

8. Rinse thoroughly with absolute alcohol.

9. Stain for half a minute in the methylene blue solution.

10. Rinse with absolute alcohol.

11. Stain for fifteen to thirty seconds in Solution D keeping the slides in motion by rocking.

12. Dehydrate thoroughly with absolute alcohol; clear in xylol and mount in balsam or cristalite.

Results:

Nuclei are stained greyish blue, while cytoplasm is yellowish; uric acid crystals are deep greenish blue; monosodium urate, brilliant green.

CELESTIN BLUE - CHROMOTROPE 2R
(Lendrum)

A substitute for haematoxylin-eosin, for simple diagnostic or photographic purposes, emphasizing the staining of collagen and reticulum

Solutions required:

A. Celestin blue (as solution B, page 89)

B. Alcohol 70% .. .. .. 100 ml.
Hydrochloric acid, conc. .. .. 2 ml.

C. Phosphomolybdic acid 1%

D. Chromotrope 2R 1% in absolute alcohol

Technique:

1. Fix tissues in Zenker or Bouin exactly as described on page 89, stage 1).
2. Stain section in the celestin blue solution for fifteen minutes.
3. Remove any cytoplasmic staining with solution B.
4. Wash with water for one minute.
5. Mordant with 1% phosphomolybdic acid solution for one to two minutes.
6. Wash well with water.
7. Dehydrate; then stain for two minutes in the chromotrope 2R solution.
8. Dehydrate; clear in xylol, and mount in D.P.X.

Results:

Nuclei, bluish purple. Cytoplasm, pink; collagenous elements, bright red.

Note: For photographing this stain the best filters to use are those giving a spectral transmission of 5,600 to 6,000 A.U.

Reference: Lendrum, A. C. (1935), *J. Path. and Bact.*, 40, 415–6, " Celestin blue as a nuclear stain ".

CELESTIN BLUE - ORCEIN - LIGHT GREEN
(Lendrum)

For breast carcinoma and skin lesions

Solutions required:

A. *Rubens - Duval Orcein:*

Orcein	0·1 gm.
Alcohol 70%	100 ml.
Nitric acid, conc.	2 ml.

B. Celestin Blue .. .. .. 0·5 gm.
Iron alum 5% .. .. .. 100 ml.
(prepared with cold distilled water)

Shake the dye with the iron alum solution in a flask; then boil for three minutes.

Allow to cool; filter; then add:

Sulphuric acid, concentrated ..	2 ml.
Glycerine	14 ml.

C. Eosin yellowish aqueous 1% .. 2 volumes
Gallic acid 0·5% aqueous .. 1 volume

D. Phosphomolybdic acid 1% aqueous.

E. *Masson's Light Green:*

Light green 2% aqueous	100 ml.
Glacial acetic acid	2 ml.

Technique:

1. Fix tissues in Zenker or Bouin; if the latter is used then picric acid must be removed by washing de-waxed and dehydrated sections on slides with saturated lithium carbonate solution. If Zenker is employed; then mercuric precipitate must be removed after fixation by the standard technique.

2. Wash; dehydrate; clear; embed in paraffin wax as usual.

3. Fix sections to slides; remove paraffin wax with xylol.

4. Pass through descending grades of alcohol down to distilled water in the usual manner.

5. Stain for one half to two hours in Solution A, in a stoppered grooved staining jar in the incubator or for twenty-four hours at room temperature.

6. Rinse well with distilled water.

7. Stain the nuclei with celestin blue (Solution B) for fifteen minutes.

8. Wash with running water for twenty minutes.

9. Stain muscle and epidermis for two minutes with eosin (Solution C).

10. Decolorize the collagen somewhat in water or in 30% alcohol.

11. Immerse in phosphomolybdic acid for two minutes.

12. Wash well in distilled water.

13. Stain collagen with the light green solution.

14. Dehydrate rapidly.

15. Clear in xylol; mount in D.P.X.

Results:

Elastin, light brown. Nuclei, bluish purple. Muscle and epidermis, red. Collagen, green.

From *J. Path. and Bact.*, 40, 415–6, 1935 Lendrum, A. C. " Celestin blue as a nuclear stain."

CHLORAZOL BLACK

A general-purpose stain, which can be used for whole tissues as well as for sections. The stain requires no mordant or differentiation, and it may be employed in aqueous or alcoholic solution. A saturated solution in 70% alcohol stains ordinary sections in fifteen to thirty minutes; the stain does not fade.

The stain is particularly suitable for staining embryo; kidney; intestine for demonstrating epithelial cells; chromatin; nucleoli; muscle fibres.

Solution required:

Chlorazol Black, saturated in 70% alcohol.

Technique:

1. Tissues should be fixed in Zenker and embedded in paraffin wax.

2. Bring sections down to 70% alcohol and remove mercuric precipitate in the usual manner.

3. Stain in a freshly prepared, unfiltered, saturated solution of Chlorazol Black in 70% alcohol for five to ten minutes.

4. Drain off excess dye; dehydrate; clear in xylol and mount.

Results:

Embryo, epithelial cell tissues, outlined in black. Chromatin, black. Nuclei, black. Muscle fibres, intense black. Lymphocytes, intense black. Blood cells, yellowish green. Cytoplasm, greenish grey.

Kidney and intestine, varying shades of green, grey and black. Blood cells, light green.

Nuclei and chromosomes are stained black; cytoplasm and secreted products grey; chitin, green; glycogen, red.

Notes:

(*a*) Benzl alcohol may also be used as a solvent, in which case results are somewhat different.

(*b*) If it is desired to differentiate chlorazal black, dilute "Milton" (a proprietary antiseptic) may be used for the purpose.

(*c*) The stain may be incorporated with Lactophenol.

CONGO RED

For Amyloid in tissues

Solutions required:

A. Congo Red 1% in distilled water.

B. Lithium carbonate saturated aqueous

C. Delafield or Ehrlich haematoxylin.

Technique:

1. Formalin or alcohol-fixed material may be embedded in Celloidin or in paraffin wax, or frozen sections may be employed.

2. Sections are mounted on slides and brought down to distilled water as usual.

3. Stain in the Congo Red solution for ten to thirty minutes.

4. Immerse in the lithium carbonate solution for fifteen seconds.

5. Decolorize in 80% alcohol until stain ceases to come away in clouds.

6. Wash in running water for fifteen minutes; then stain with Ehrlich or Delafield haematoxylin for five to ten minutes.

7. Wash in water; dehydrate in the usual manner; clear in xylol and mount.

Note: If Celloidin sections are used dehydration should be carried out with Isopropyl alcohol in place of ethyl alcohol.

Results:

Amyloid, red; nuclei, blue.

CONGO RED - ANILINE BLUE - ORANGE G

For elastic fibres

Solutions required:

A. Aluminium chloride, 2% aqueous.

B.

Congo Red	2 gm.
Sodium citrate	2·5 gm.
Glycerin	1 ml.
Distilled water	97 ml.

C.

Aniline Blue, aqueous	1·5 gm.
Orange G	2·25 gm.
Resorcinol	3 gm.
Phosphomolybdic acid 1% aqueous	100 ml.

Technique:

Tissues should be fixed in 10% formalin, and frozen sections should be employed.

1. Wash sections in water; then immerse them in Solution A for ten minutes.

2. Wash with water and drain; then stain in the Congo Red solution for ten minutes.

3. Wash with tap water; then plunge the slide into a dish of tap water and agitate it there for ten seconds.

4. Wash again with tap water; then stain from five to ten minutes in the Aniline Blue-Orange G solution (Solution C above).

5. Rinse carefully in tap water; drain well and blot.

6. Dehydrate in absolute alcohol; clear in origanum oil; wash in xylol and mount.

Results:

Elastic fibres, bright red; fibrin, dark blue.

CONGO RED - EHRLICH HAEMATOXYLIN

For eleidin and keratohyalin

Solutions required:

A. Congo Red 0·05% aqueous.

B. Ehrlich haematoxylin.

Technique:

1. Material should be fixed in absolute alcohol and embedded in paraffin wax.

2. Sections not more than 5μ thick are mounted on slides and brought down to distilled water as usual.

3. Stain for five to ten minutes in the Congo Red solution.

4. Rinse in distilled water; then stain for five to ten minutes in Ehrlich haematoxylin.

5. Blue in tap water in the usual manner.

6. Dehydrate; clear in xylol and mount.

Results:

Eleidin is stained red, while nuclei and keratohyalin are blue.

CRESYLFAST VIOLET - TOLUIDINE BLUE - THIONIN (EHRLICH)

A non-fading tri-basic stain for nerve cells and Nissl granules, in normal and pathological tissues

Solutions required:

A.	Cresylfast violet, CNS	2 gm.
	Toluidine blue	1 gm.
	Thionin (Ehrlich)	0·5 gm.
	Ethyl Alcohol 30%	200 ml.

B. Distilled water .. .. .. 200 ml.
Sulphuric or nitric acid, conc. .. 0·5 ml.

Technique:

1. Formalin fixed material is embedded in paraffin wax, and sections, 4μ in thickness are fixed to slides with glycerin albumen.

2. Remove wax with xylol.

3. Rinse with absolute alcohol.

4. Pass through 95% alcohol.

5. Pass through 80% alcohol.

6. Immerse slides for five to ten seconds in the staining solution at 80–90° C.

7. Differentiate for one second in solution B.

8. Dip and agitate slides in a beaker of cold distilled water for one second.

9. Differentiate further in 80% and 95% alcohol for one to two seconds in each.

10. Immerse in 80% alcohol for one second.

11. Dip and agitate the slides in the still warm solution A for one to two seconds.

12. Return to 80% alcohol for one second.

13. Repeat steps 11 and 12.

14. Rinse in distilled water.

15. Dehydrate by immersing for one second in each of 80%, 95% and absolute alcohol.

16. Immerse in xylol for one minute.

17. Immerse in a fresh lot of xylol for three minutes.

18. Mount and examine.

Results:

Neurons stand out distinctly against a pale background, and can be followed for a considerable distance. The cytons are stained dark purple emphasizing the blue tint, while the dendrite and axon processes and endings present a somewhat lighter shade, bluish to reddish. Granules in the cell body as well as in the protoplasm

processes appear purple or reddish. Nuclei and nucleoli are well differentiated.

Reference: Spoerri, Rosette (1948), *Stain Tech.*, **23**, 133–5.

DAHLIA ACETIC (Ehrlich)

For mast cell granules in sections

Solution required:

Distilled water	100 ml.
Absolute alcohol	50 ml.
Glacial acetic acid	12·5 ml.
Dahlia	10 gm.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool; then filter.

Technique:

1. Fix tissues in absolute alcohol and embed in Celloidin.
2. Immerse sections in the staining solution for twelve hours.
3. Differentiate in 95% alcohol.
4. Clear in origanum oil and mount in balsam or in cristalite.

Results:

Granules of mast cells are stained reddish violet.

DOPA REAGENT

For melanoblasts

Solutions required:

A.

Dopa reagent (3 : 4-dihydroxy-phenylalanin)	0·2 gm.
Cold distilled water	200 ml.

Note: This solution, which deteriorates fairly rapidly at normal temperatures, should be kept in a refrigerator. When the solution turns dark red it is useless and should be discarded.

B.

Buffer tablet pH 7·4	1 tablet
Distilled water (cold)	100 ml.

C. Solution A .. .. .. 25 ml.
Solution B .. .. .. 8 ml.

Note: This solution must be freshly prepared as required.

Technique:

Tissues should be fixed not longer than two to three hours in 5% formalin, or frozen sections of fresh material may be employed.

1. Rinse with distilled water from four to five seconds; then immerse in Solution C for three to four hours, controlling under the microscope at intervals, until melanoblasts are stained black.

Note: This solution is likely to overstain if it becomes sepia brown.

2. Wash with distilled water; dehydrate; clear; mount.

Result:

Melanoblasts, black.

Note: After dehydration (in Stage 2) 1% crystal violet in absolute alcohol may be employed as a counterstain, if desired.

ELASTIN STAIN

(Weigert)

Any fixative may be used except Susa, and tissues may be embedded in paraffin wax or in Celloidin or in L.V.N.

Preparation of the Staining Solution:

Triturate 1 gm. of Weigert elastin stain and 5 gm. clean, dry silver sand with 100 ml. absolute alcohol and 2 ml. pure hydrochloric acid until all the stain has gone into solution; then filter.

Note: The staining solution deteriorates after two or three weeks.

The nuclei may be stained with Orth's lithium carmine prior to the following procedure if no other counterstain is desired.

Technique:

1. Sections are brought down to 90% alcohol and stained one half to twelve hours according to depth of staining desired. The slides

should be stained in a jar or in a Petri dish, sections face downwards, to prevent a deposit forming on the sections.

2. Wash off excess stain with 95% alcohol, and if necessary differentiate in acid alcohol for a few minutes.

3. Wash quickly with 70% alcohol; then thoroughly with water.

4 Counterstain with Van Gieson, Ehrlich haematoxylin or Safranin for about five minutes.

5. Differentiate, if necessary, in 95% alcohol.

6. Dehydrate; clear in xylol and mount.

Note: If Celloidin or L.V.N. sections are used clear in origanum oil or in terpineol after 95% alcohol.

Results:

Elastic fibres, dark blue or black. Nuclei, brilliant red (if Orth's carmine is used) or bluish black (with haematoxylin). Collagen, pink to red; other tissue elements, yellow (if Van Gieson is used).

ELASTIN STAIN (Sheridan)

This stain has an advantage over Weigert's elastin stain in that the solution may be kept for reasonably long periods without deterioration.

The staining procedure is the same as for Weigert's elastin stain.

Results:

Elastic fibres are stained green to greenish black.

ELASTIN - TRICHROME STAIN

For the demonstration of elastic, smooth muscle and collagenic fibres with equal clarity, particularly in the walls of blood vessels

Solutions required:

A. *Weigert's elastin stain*

Weigert's elastin stain powder ..	1 gm.
Hydrochloric acid, conc., pure ..	2 ml.
Absolute alcohol	100 ml.

Dissolve the stain by boiling for two minutes in a flask, plugged lightly with cotton-wool, on a water bath. Allow to cool; then filter; make the volume up to 100 ml. with absolute alcohol; then add the acid. Alternatively the solution may be prepared as described on p. 96.

Note: This solution deteriorates after three or four weeks.

B. Ehrlich haematoxylin.

C. *Ponceau-acid fuchsin* (*Masson*):

Acid fuchsin	0·3 gm.
Ponceau de xylidine	0·7 gm.
Distilled water	100 ml.
Glacial acetic acid	1 ml.

D. Phosphotungstic acid 3% aqueous.

E. Light Green 1% aqueous.

Technique:

1. Paraffin sections are mounted on slides and brought down to distilled water in the usual manner; then immersed in Weigert's elastin stain in a staining jar for one hour.

2. Wash rapidly in acid alcohol; then dehydrate and differentiate in absolute alcohol until the sections appear only faintly red.

3. Immerse in 70% alcohol, followed by distilled water.

4. Stain in Ehrlich haematoxylin for eight to ten minutes; then differentiate in water for five minutes.

5. Stain in Ponceau-acid fuchsin for five minutes.

6. Wash thoroughly in 3% phosphotungstic acid; then immerse in the phosphotungstic acid for ten minutes.

7. Wash thoroughly in distilled water; then stain with Light Green for two to five minutes; then without washing:

8. Flood the preparation with 1% acetic acid and allow it to act for three minutes; pour off excess; then without washing:

9. Dehydrate; clear; mount in Damar xylol.

Results:

Elastic tissue stained blue-black; smooth muscle, red; collagen green.

EOSIN AZUR 2 - HAEMATOXYLIN (Maximow)

For demonstration of inflammatory changes in haemopoietic tissues

Solutions required:

A. Azur 2 eosin .. .. .. 0·1 gm.
Distilled water .. .. .. 100 ml.
Heat to boiling point, then allow to cool.

B. Solution A (as above) .. .. 10 ml.
Distilled water .. .. .. 50 ml.

C. Ehrlich haematoxylin.

Technique:

1. Formalin-fixed material (sections or smears) are stained from five to ten minutes with Ehrlich haematoxylin.

2. Pour off excess stain; immerse the preparation in tap water until it appears blue to the naked eye; then wash thoroughly with distilled water and drain well.

3. Stain from eighteen to twenty-four hours in Solution B (as above) in a staining jar. If a staining jar is not available, place the slide, resting face downwards, on two pieces of thin glass rod, so that any precipitate formed is not deposited on the preparation.

4. Differentiate in 95% alcohol until dense blue clouds cease to come away from the preparation, and the red corpuscles and collagen are pink.

5. Immerse the preparation in three changes of absolute alcohol, followed by two changes of xylol; then mount.

Results:

Cartilage stained purple; basophil leucocytes and mast cell granules, purple to violet; nuclei, blue; erythrocytes, pink; cytoplasm, pink to blue; eosinophil granules and secretion granules, pink.

ETHYL VIOLET - BIEBRICH SCARLET (Bowie)

For pepsinogen granules in gastric mucosa

Solutions required:

A. *Stock solution.*

Ethyl violet-Biebrich scarlet 1% in 20% alcohol.

B. *Staining solution.*

Stock solution (as above)	0·5 ml.
Alcohol 20%	100 ml.

Technique:

1. Tissues should be fixed in Regaud's fluid, washed in running water, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Fix sections to slides, dewax and pass through descending grades of alcohol down to distilled water.

3. Stain for twenty-four hours in Solution B in a covered staining jar.

4. Drain and wipe off excess liquid.

5. Differentiate for about half an hour with a mixture consisting of equal volumes of clove oil and xylol, controlling by examination under the microscope at intervals.

6. Wash well with several changes of xylol; mount in cristalite.

Results:

Pepsinogen of the pepsin-forming cells is stained violet, while parietal cells are red.

FEULGEN FUCHSIN

For chromatin in animal cells

Solutions required:

A. N/1 hydrochloric acid.

B. Distilled water .. .. .. 200 ml.

Boil; allow to cool to about 70° C.; then add:
Basic fuchsin .. .. .. 1 gm.

When dissolved, raise to boiling point; then allow to cool to 50° C. and add:

2 ml. pure hydrochloric acid conc. and
2 gm. potass. metabisulphite.

Allow to stand twenty-four hours then add 1 gm. decolorizing carbon; shake well; filter. The solution if properly prepared will be colourless.

C. Pure hydrochloric acid, conc. .. 2 ml.
Potass. metabisulphite .. .. 2 gm.
Distilled water .. .. .. 200 ml.

D. Light green 0·25% in 90% alcohol.

Technique:

Tissues are fixed in Zenker or Helly for six to twelve hours; then washed in running water for twelve to twenty-four hours. They should not be treated with iodine-alcohol.

1. Sections are transferred from distilled water to a jar of Solution A at 60° C. for five to fifteen minutes; then rinsed in distilled water.

2. Transfer for one to one and a half hours to a jar of Solution B at room temperature.

3. Transfer for two to three minutes in each of three successive lots of Solution C; then wash thoroughly in distilled water.

4. Counterstain for about two to five minutes in Solution D.

5. Dehydrate; clear and mount.

Results:

Chromatin, purple. All other elements, transparent green.

FONTANA STAIN

For argentaffine granules

Solutions required:

A. Silver oxide (Fontana).

B. Sodium thiosulphate 5% aqueous.

Technique:

1. Tissues are fixed in 10% neutral formalin, washed, dehydrated in alcohol, cleared in cedarwood oil, and embedded in paraffin wax as usual.

2. Fix sections to slides, bring down to distilled water and wash thoroughly in two or three changes of neutral, freshly distilled water.

3. Immerse in the silver oxide (Fontana) solution for twelve to twenty-four hours in the dark in a covered, scrupulously clean vessel.

4. Wash in neutral, freshly distilled water for one minute.

5. Immerse for one minute in the sodium thiosulphate.

6. Immerse in tap water for ten minutes.

7. Counterstain, if desired in carmalum.

8. Dehydrate; clear in xylol and mount.

Results:

Argentaffine granules, black.

FONTANA STAIN - SILVER NITRATE

For reticular and collagen fibres

Solutions required:

A. Strong ammonia solution (sp. gr. 0·880) .. .. 10 ml.
Distilled water .. .. .. 90 ml.

B. Potassium permanganate 0·5% aqueous.

C. Oxalic acid 1·5%.

D. Silver nitrate 5% aqueous.

E. Fontana stain (silver oxide solution)

F. Aniline oil.. .. .. .. 1 volume
Xylol .. .. 1 volume

Technique:

1. Frozen sections not thicker than 10μ are fixed in 10% formalin and afterwards washed in three changes of water for fifteen minutes in each.

2. Immerse in Solution A at 60° C. for fifteen minutes, in an oven.

3. Rinse well in three changes of distilled water.

4. Immerse in the potassium permanganate for three or four minutes.

5. Rinse with distilled water for about ten or twenty seconds.

6. Decolorize with the oxalic acid solution until the brown colour just disappears; then wash well in distilled water.

7. Immerse in silver nitrate solution in the dark for an hour.

8. Wash well with two changes of distilled water in the dark.

9. Immerse in Fontana's stain for fifteen minutes at 60° C. in the dark.

10. Wash rapidly in three changes of distilled water.

11. Immerse in 30% formalin for two or three minutes at 60° C.

12. Wash thoroughly in running tap water; then transfer to slides.

13. Blot away excess water.

14. Dehydrate with two changes of absolute alcohol.

15. Clear in the aniline-xylol (Solution F, above).

16. Wash with xylol; mount in dammar xylo

Results:

Reticulum is stained black, while collagen is brown.

N.B.: Sections must be handled with glass needles throughout this technique, as contact with metal instruments will ruin the preparations.

GALLOCYANIN - ORCEIN - ACID ALIZARIN BLUE - ALIZARIN VIRIDINE

A general stain for animal tissues

Solutions required:

A. Gallocyanin .. .. .. 0·1 gm.
Chrome alum 5% aqueous .. 100 ml.

Boil for ten minutes. Allow to cool; then make up the volume to 100 ml., filter and add five or six drops of formalin.

B. Orcein 0·5% in 70% alcohol .. 99 ml.
Hydrochloric acid conc. .. 1 ml.

C. Acid Alizarin Blue .. .. 5 gm.
Aluminium sulphate 10% aqueous 100 ml.

Boil for ten minutes. Cool and filter. Make up the volume to 100 ml. with distilled water and add five or six drops of formalin.

D. Phosphomolybdic acid 5%.

E. Alizarin viridin .. .. .. 0·2 gm.
Buffer solution pH 5·8 .. 100 ml.

Technique:

1. Fix tissues in 10% formalin and embed in paraffin wax in the usual manner.

2. Fix sections to slides and take down to distilled water as usual.

3. Stain nuclei intensely by immersing the slides in the gallocyanin solution in a staining jar, examining the preparations under the microscope at intervals over a period of twenty-four hours, to ascertain the depth of staining.

4. Wash with two changes of distilled water.

5. Stain elastic fibres in the orcein solution for ten minutes to half an hour in a grooved, covered staining jar.

6. Wash well with distilled water.

7. Stain muscle in the acid alizarin blue solution for seven minutes.

8. Wash with distilled water.

9. Differentiate in the phosphomolybdic acid solution for about thirty minutes, controlling by examination under the microscope at intervals.

10. Wash with two changes of distilled water.

11. Stain collagen in the alizarin viridin for seven minutes.

12. Drain and blot thoroughly but carefully.

13. Rinse with 96% alcohol; followed by carbol xylol.

14. Wash well with two or three changes of xylol, and mount.

Results:

Nuclei, dark brown. Muscle and epithelium, pale violet. Erythrocytes and elastic fibres are stained a rich brown, while mucus, collagen are in varying shades of green; myelin sheaths, pink; and axis cylinders, dark blue.

GIEMSA STAIN

For malarial parasites, rickettsia, etc.

Solution required:

Giemsa stain .. .. .. 1 ml.
Distilled water, buffered to pH 7·2 20 ml.
N.B.: This mixture should be freshly prepared immediately before use.

Technique:

1. Fix small pieces of tissue in 10% formalin, Regaud or Zenker.

2. Dehydrate; clear and embed.

3. Bring down paraffin sections to distilled water in the usual manner.

4. Stain for eighteen to twenty-four hours in the diluted Giemsa (as above).

5. Wash in distilled water; differentiate quickly in 0·5% acetic acid until the section is pink; then wash with distilled water.

6. Blot and dry in air and mount.

Results:

Nuclei are stained dark red; erythrocytes, pink. Malaria parasites, bluish red with red chromatin.

GIEMSA - WRIGHT STAIN

A permanent stain for differentiating the structures, particularly Nissl bodies and cytons, of the spinal cord

Solution required:

Wright's stain	5 volumes
Giemsa stain	1 volume

Technique:

1. Material should be fixed in neutral formalin 10%.

2. Wash, dehydrate, clear, and embed in paraffin wax in the usual manner.

3. Fix sections to slides; de-wax; pass through the usual descending grades of alcohol, down to distilled water.

4. Flood the sections with a measured volume of the above staining solution and allow it to act for two minutes.

5. Add an equal volume of distilled water and mix with stain by rocking the slides gently. Allow this diluted stain to act for two minutes.

6. Pour off excess stain and immerse the slides in fresh distilled water for one minute.

7. Transfer immediately into 80% alcohol and leave therein for fifteen seconds.

8. Dehydrate rapidly in 95% and absolute alcohol.

9. Clear in xylol and mount.

Results:

Cytons and Nissl granules are stained deep blue. Nuclei of blood-vessel structures and neuroglia are light blue. Elastic fibres of blood vessels, deep blue. Erythrocytes, pink. Neuroglia fibres, light red.

Note: The proportion of the Giemsa stain regulates the intensity of the cyton stain.

Reference: Hanburg, L. (1935), *Science*, **81**, 364–5.

GOLD CHLORIDE - SUBLIMATE (Cajal)

For neuroglia fibres; for astrocytes in central nervous system

Solutions required:

A. Neutral formalin .. .. .. 15 ml.
Ammonium bromide .. .. 2 gm.
Distilled water .. .. .. 85 ml.

B. Gold chloride (brown or yellow) 1% aqueous.

C. Mercuric chloride 5% aqueous.

D. Sodium hyposulphite 10% aqueous.

Technique:

1. Fresh pieces of tissue are fixed for two to twenty-one days in Solution A.

2. Frozen sections are cut 15 to 30μ thick.

3. Rinse in several changes of distilled water.

4. Immerse sections, flattened out and not lying on top of one another, for three to four hours in a freshly prepared mixture consisting of:

Solution B .. .. .. 5 ml.
Solution C .. .. .. 5 ml.
Distilled water .. .. .. 30 ml.

(*Note:* 3 ml. of the mixture is required for each section)

until the astrocytes are stained dark against a relatively light background; the reaction should be controlled by microscopic examination of a section while still wet.

5. Wash in distilled water; then fix in Solution D.

6. Wash thoroughly in tap water.

7. Dehydrate; clear and mount.

Results:

Astrocytes, black. Nerve cells, red. Nerve fibres, unstained. Background, light brownish purple, or unstained.

GOLGI METHOD (Rapid)

For nerve cells

Solutions required:

A. Potass. bichromate 3% .. .. 40 ml.
Osmic acid 1% .. .. .. 10 ml.
This solution must be freshly prepared.

B. Silver nitrate 0·75%.

Technique.

1. Immediately the animal is killed, tissues are cut into slices about 2 mm. thick and fixed in Solution A for one to three days depending upon the size of the pieces.

2. After removing excess fixative by blotting, the tissues are rinsed in Solution B until no more precipitate is formed.

3. Transfer to a fresh lot of Solution B and leave for two days or longer.

4. With a camel-hair brush carefully brush the precipitate from the surface of the tissue; then wash well in distilled water.

5. Dehydrate by immersing for one to four hours, depending upon the size of the pieces, in each of the following: two lots of 95% alcohol; two lots of absolute alcohol; one lot of ether-alcohol (equal vols. ether and absolute alcohol).

6. Embed in Celloidin or L.V.N.

7. Sections 60–100μ are mounted on slides and covered with Canada balsam in benzol or cristalite, without cover glasses (*see* L.V.N. technique, page 26).

Results:

Background, dull yellow. Nerve cells and their processes, black. Blood vessels, black.

GRAM'S IODINE

For bacteria in sections

Solutions required:

A. Carbol gentian violet.
B. Gram's iodine.

C. Carbol fuchsin (Ziehl Neelsen) .. 1 volume
Distilled water .. .. .. 9 volumes

D. Picric acid, saturated, aqueous.

Technique:

1. Pieces of tissue are fixed in 10% formalin; dehydrated; cleared and embedded in paraffin wax.

2. Fix sections to slides; de-wax and take down to distilled water in the usual manner.

3. Stain in Solution A for about two minutes.

4. Pour off excess stain and without washing add Gram's iodine and allow the stain to act for one minute.

5. Differentiate in pure acetone until colour ceases to come out of the sections.

6. Counterstain in the carbol fuchsin (Solution C) for about a minute.

7. Pour off excess stain, and drain, without allowing the sections to dry; then without washing:

8. Cover the sections with the picric acid solution, pouring off after one half to one minute.

9. Dehydrate and clear with pure acetone for about fifteen seconds.

10. Clear in xylol and mount.

Results:

Gram-positive organisms are stained violet, while Gram-negative are red. Nuclei are stained pink, while cytoplasm is yellow.

HAEMALUM - EOSIN

For demonstrating collagenous tissue

Solution required:

A. Haemalum (Mayer).

B. Eosin, yellowish 0·2% in 20% alcohol.

Technique:

1. Paraffin sections are fixed to slides, de-waxed and taken down through descending grades of alcohol to distilled water in the usual manner.

2. Stain for five to ten minutes with the haemalum solution, examining under the microscope at intervals until a satisfactory degree of staining has been achieved.

3. Rinse for a few seconds in tap water.

4. Stain for one or two seconds with the eosin solution.

5. Rinse for a few minutes in running tap water.

6. Pass through 70%, 90% and absolute, alcohol.

7. Clear in xylol and mount.

Results:

Collagen, deep pink. Smooth muscle, pink. Cytoplasm, pale pink. Nuclei, blue.

HAEMATOXYLIN - AZOPHLOXINE

For muscle, connective tissue, ganglion cells, etc.

Solutions required:

A. *Lavdowsky's Fixative:*

Formalin (40% formaldehyde)	10 ml.
Glacial acetic acid	2 ml.
Alcohol 95%	50 ml.
Distilled water	40 ml.

B. Haematoxylin (Delafield, Harris or Ehrlich)

C. Azophloxine 0·2 gm.
Acetic acid 0·2% 100 ml.
Add about 1 ml. chloroform as a preservative.

D. Acetic acid 0·2% aqueous.

E. Orange G. 2 gm.
Phosphotungstic acid 4 gm.
Distilled water 100 ml.
Fast Green FCF 0·2 gm.

Technique:

1. Fix material in Lavdowsky's mixture or in 10% formalin and embed in paraffin wax.
2. Stain in Harris, Ehrlich or Delafield Haematoxylin for five to ten minutes.
3. Blue in tap water or 1% lithium carbonate solution.
4. Stain in the azophloxine solution for two minutes.
5. Rinse in 0·2% acetic acid.
6. Stain in the orange G-fast green solution for one minute.
7. Differentiate for about five minutes with 0·2% acetic acid.
8. Rinse in distilled water.
9. Remove excess distilled water by draining and blotting round the edges of the sections carefully, but do not allow to dry completely.
10. Dehydrate directly with two or three changes of absolute alcohol, or with cellosolve.

Note: Absolute and 70% alcohol should be avoided as they have a tendency to remove the azophloxine.

Results:

Connective tissue, green. Striated muscle, brick red. Smooth muscle, reddish violet. Nerves, blue-grey. Ganglion cells, violet. Erythrocytes, orange. Cardiac conductive tissue is easily distinguishable from cardiac muscle as it takes a lighter shade of staining.

Note: Azophloxine is used here as a substitute for ponceau de xylidine in Goldner's modification of Masson's technique.

The stain is also suggested in place of Eosin as a counterstain for use with haematoxylin. The advantages of using azophloxine are that it gives clear and delicate pictures and it does not overstain, if the recommended procedure is followed. When azophloxine is to be used merely as a counterstain for haematoxylin the procedure is as follows:

I. Proceed as steps, 1, 2, 3, 4, and 5 (above).
II. Rinse in distilled water.
III. Dehydrate with three changes of absolute alcohol; then clear and mount.

Reference: Halper, M. H. (1954, November), *Stain Tech.*, **29**, no. 6, 315–7.

HAEMATOXYLIN - BASIC FUCHSIN

For haemofuscin, melanin and haemosiderin in animal tissues

Solutions required:

A. Haematoxylin (Ehrlich).

B. Basic fuchsin 0·5% in 50% alcohol.

Technique:

Tissues may be fixed in Zenker or in absolute alcohol or in 10% formalin. Paraffin or Celloidin sections may be employed. If Zenker's fixative is used it will be necessary to remove mercury deposits in the usual manner.

1. Stain for five to ten minutes in Ehrlich haematoxylin.

2. Wash well in tap water, then several times in distilled water.

3. Stain from five to twenty minutes in the basic fuchsin solution; then pour off excess stain and wash well in distilled water.

4. Differentiate in 95% alcohol; then dehydrate in absolute alcohol; clear in xylol and mount in balsam.

Results:

Nuclei, blue; melanin and haemosiderin remain unstained in their natural brown colours; haemofuscin, bright red.

HAEMATOXYLIN (DELAFIELD) - EOSIN

For general staining

Solutions required:

A. Delafield haematoxylin.

B. Eosin, yellowish, 1%, aqueous.

Technique:

1. Tissues should be fixed in Zenker, Bouin or 10% formalin and embedded in paraffin wax.

2. Sections are brought down to distilled water; then stained with Delafield haematoxylin for ten minutes.

3. Wash and immerse in tap water for about five minutes until the section appears blue to the naked eye.

4. Wash rapidly with distilled water; then stain for one to two minutes with 1% eosin yellowish, aqueous.

5. Wash quickly with distilled water; then dehydrate with 95% and absolute alcohol.

6. Clear in xylol and mount.

Results:

Nuclei are stained blue; cytoplasm, pink.

HAEMATOXYLIN (Ehrlich)

For keratohyalin

Solutions required:

A. Haematoxylin (Ehrlich).

B. Potass. permanganate 0·1%.

Technique:

1. Material should be fixed in 10% formalin and embedded in paraffin wax.

2. Fix sections to slides and bring down to distilled water as usual.

3. Stain in Ehrlich haematoxylin for ten minutes.

4. Pour off excess stain; then rinse and blue in tap water.

5. Immerse in potassium permanganate 0·1% for ten seconds; then wash well with water.

6. Dehydrate; clear; mount in balsam.

Results:

Keratohyalin is stained blue-black while the other elements are unstained or faintly stained.

HAEMATOXYLIN (Ehrlich)

For demonstrating sodium urate in animal tissue

Solution required:

Ehrlich or Delafield haematoxylin.

Technique:

1. Fix tissues in absolute alcohol and embed in Celloidin.
2. Stain for five to ten minutes in the haematoxylin solution.
3. Blue in saturated lithium carbonate solution.
4. Rinse quickly in distilled water.
5. Dehydrate with 95% alcohol.
6. Clear in terpineol.
7. Mount in balsam.

Results:

Sodium urate crystals and nuclei, deep blue.

HAEMATOXYLIN (Ehrlich) - EOSIN

A general stain for animal tissues

Solutions required:

A. Haematoxylin (Ehrlich).

B. Eosin, aqueous, yellowish 1% in tap water.

Technique:

1. Sections are mounted on slides and brought down to distilled water in the usual manner, any mercurial deposit from the fixative being removed by the standard technique.

2. Stain from two to ten minutes in haematoxylin (Ehrlich) depending upon the " ripeness " of the stain (a well-ripened haematoxylin will act much more rapidly than a recently-prepared solution).

3. Rinse in water; then " blue " in tap water; that is, immerse the preparation in tap water from two to ten minutes or until the preparation appears blue to the naked eye.

4. The preparation should now be examined, while still wet,

under the microscope and if the nuclei are not stained a bright and transparent blue and the cytoplasm (except mucin and basophile granules of mast cells, etc.) is not colourless, rinse in water and repeat the staining and bluing process.

If, on the other hand, overstaining has taken place, immerse the preparation in 0·5% HCl for five to thirty seconds; then immediately rinse in water, " blue " in tap water and again examine under the microscope; if the sections are still overstained repeat the treatment with HCl; rinse and " blue " in tap water again.

5. Wash well with water; then stain from two to five minutes in eosin solution.

6. Rinse quickly in water and examine the section rapidly, while still wet, under the microscope to ensure that the depth of the counterstain is sufficient. The cell cytoplasm, collagen, connective tissue fibres, erythrocytes, etc., should be stained a bright transparent pink. It is advantageous to overstain somewhat with the eosin, as subsequent dehydration in the alcohols will remove the excess eosin.

7. Dehydrate by passing rapidly through 70%, 90% and absolute alcohol.

8. Clear in xylol; mount in balsam.

Results:

Nuclei are stained dark blue; karosomes, dark blue; plasmasomes, red; cytoplasm (except when basophile) is stained in varying shades of pink; muscle and collagen fibres, pink; elastic fibres (when thick), pink; erythrocytes, thyroid colloid and keratin, bright pink.

HAEMATOXYLIN - FLUORCHROME (Kultschitzky-Pal)

For myelin sheaths. Particularly suitable for demonstrating very fine fibres in cerebal cortex, etc.

Solutions required:

A. *Weigert's rapid fixative:*

Fluorchrome	2 gm.
Potassium bichromate	5 gm.
Distilled water	100 ml.

Add the potassium bichromate to the water; boil; add the fluorchrome; cool; then filter.

B. Kultschitzky's haematoxylin solution.

C. Lithium carbonate, saturated, aqueous .. .. .. 100 ml.
Potassium ferricyanide 1% aqueous .. .. .. 10 ml.

Technique:

1. Slices of formalin-fixed tissue not more than 5 mm. thick are immersed in Weigert's rapid fixative from four to seven days.

2. Wash in running water for four to six hours.

3. Dehydrate and embed in Celloidin.

4. Stain sections in Kultschitzky's haematoxylin from twelve to twenty-four hours.

5. Differentiate for several hours in Solution C controlling under the microscope at intervals of one half to one hour, until the white matter is stained blue-black, and the grey matter is stained yellowish.

Note: For human spinal cord differentiation takes up to twelve hours. Human cerebral cortex requires about four hours.

6. Wash well in running water.

7. Dehydrate and clear by the standard Celloidin method.

8. Mount in cristalite.

Results:

Myelin sheaths are stained black, while ground-substance is yellow.

HAEMATOXYLIN - GENTIAN VIOLET - IODINE

For demonstrating Gram-positive bacteria and fibrin in sections

Solutions required:

A. Haematoxylin (Delafield).
B. Aniline gentian violet
C. Lugol's iodine.
D. Aniline oil.. .. .. .. 20 ml.
Xylol .. .. 10 ml.
E. Erythrosin 5% in absolute alcohol.

Technique:

1. Tissues may be fixed in 10% formalin or in Zenker, if the latter is used then mercuric precipitates must be removed from the sections by the standard technique.

2. Fix sections to slides; de-wax with xylol and pass through descending grades of alcohol to water in usual manner.

3. Stain in the haematoxylin solution for five to twenty minutes.

4. Rinse quickly in acid alcohol.

5. Immerse in a large volume of tap water for two to five minutes.

6. Stain in the aniline gentian violet for two to five minutes.

7. Pour off excess stain, and without washing, blot the slide carefully.

8. Flood with Lugol's iodine and allow the solution to act for two to five minutes.

9. Pour off excess, and without washing, blot dry carefully.

10. Decolorize for a few seconds with Solution D.

11. Flood the preparation with erythrosin (Solution E, above) and allow the stain to act from one half to one minute.

12. Pour off excess stain and wash the preparation with Solution D.

13. Rinse well with xylol; drain off excess and blot dry carefully.

14. Mount in balsam or Cristalite or Clearmount.

Results:

Nuclei, blue. Fibrin and Gram-positive organisms, purplish blue.

HAEMATOXYLIN

(Heidenhain)

Solutions required:

A. Iron alum .. .. .. 3 gm.
Distilled water .. .. .. 100 ml
Dissolve by shaking.

B. Haematoxylin 10% in absolute alcohol (which has been ripened for three months or longer) .. 5 ml.
Distilled water .. .. .. 95 ml.

C. Eosin yellowish 1% aqueous.

Technique:

Fix in Zenker. Embed in paraffin wax.

1. Sections are brought down to distilled water, then mordanted in Solution A for one half to three hours.

2. Wash in tap water; stain one to three hours in Solution B; then rinse in tap water.

3. Differentiate in Solution A, controlling by examination under the microscope.

4. Wash in running water for five to ten minutes.

5. Stain with Solution C for one to three minutes.

6. Wash in tap water; dehydrate; then clear in xylol and mount.

Results:

Nuclei are stained black; cytoplasmic structures, pink.

HAEMATOXYLIN

For the identification of lipines

Solutions required:

A. Potassium bichromate 5% aqueous.

B. Haematoxylin solution (Ehrlich).

C. Potassium ferricyanide .. .. 2·5 gm.
Borax .. .. 2 gm.
Distilled water .. .. .. 100 ml.

Technique:

1. Tissues are fixed from twelve to twenty-four hours in 10% formalin in normal saline.

2. Wash for several hours in running water.

3. Make frozen sections and collect them in distilled water.

4. Immerse in Solution A for twenty-four to forty-eight hours at 37° C.

5. Wash in several changes of distilled water, handling the sections with care (as they become brittle after immersion in Solution A).

6. Immerse in Solution B for four to six hours at 37° C.

7. Wash in distilled water.

8. Differentiate in Solution C, controlling under the microscope, until the ground cytoplasm is changed from black to yellow. This process takes several hours.

9. Wash thoroughly in five or six changes of distilled water; then mount in glycerine jelly.

Result:

Lecithin and other lipines are stained black to *deep* blue (light blue coloration should not be taken as positive). Lipides and other tissue constituents are colourless.

HAEMATOXYLIN (Kultschitzky)

(Weigert's modification)

For finer studies of cortical architecture and for total brain sections

Solutions required:

A. *Weigert's Secondary Mordant:*

Cupric acetate neutral, normal ..	5 gm.
Fluorchrome	2·5 gm.
Distilled water	100 ml.
Boil; allow to cool; then add:	
Glacial acetic acid	5 ml.

B. Haematoxylin (Kultschitzky).

C. Lithium carbonate, saturated, aqueous .. .. .. 100 ml.
Potassium ferricyanide 1% aqueous 10 ml.

Technique:

1. After fixing material in 10% formalin mordant for four to five days in Solution A.

2. Dehydrate in ascending grades of alcohol in the usual way, and embed in Celloidin.

3. Immerse for twelve to twenty-four hours in the haematoxylin (Solution B).

4. Differentiate in Solution C from four to twelve hours, controlling by examination under the microscope at intervals, and changing the differentiating fluid three or four times.

5. Wash thoroughly in distilled water.

6. Dehydrate with 95% alcohol.

7. Clear in terpineol.

8. Drain well and blot carefully.

9. Mount in balsam.

Results:

Finest myelin sheaths are stained a deep black.

HAEMATOXYLIN - PHLOXINE - ANILINE GENTIAN VIOLET

For actinomyces in sections

Solutions required:

A. Ehrlich haematoxylin.

B. Phloxine 3% aqueous.

C. Aniline gentian violet.

D. Gram's iodine.

Technique:

1. Tissues are fixed in 10% formalin, washed, dehydrated, cleared and embedded in paraffin wax in the usual manner.

2. Fix sections to slides; de-wax and bring down to distilled water as usual.

3. Stain with Ehrlich haematoxylin five to ten minutes; then blue and wash in lithium carbonate saturated aqueous.

4. Stain for fifteen to twenty-five minutes in the phloxine solution; then wash with distilled water.

5. Stain in aniline gentian violet for about ten minutes.

6. Rinse in distilled water.

7. Immerse in Gram's iodine solution for one minute.

8. Wash in distilled water.

9. Decolorize with aniline oil until the stain ceases to come out of the sections.

10. Rinse well in several changes of xylol and mount.

Results:

Branched forms are stained blue, while clubs appear red.

HAEMATOXYLIN, PHOSPHOTUNGSTIC (Mallory)

For Pleuropneumonia organisms in sections of lung

Solution required:

Phosphotungstic acid haematoxylin (Mallory).

Technique:

1. Pieces of tissue should be fixed in Bouin, Carnoy or absolute alcohol and embedded in paraffin wax in the usual manner.

2. Fix sections to slides; de-wax, pass through descending grades of alcohol down to distilled water, as usual.

3. Stain in Mallory's phosphotungstic acid haematoxylin in a stoppered staining jar for twenty-four hours, without treatment with the usual potassium permanganate and oxalic acid solutions.

4. Pour off excess stain; drain well without washing; then carefully blot dry.

5. Dehydrate quickly in absolute alcohol.

6. Clear in xylol and mount.

Results:

The organisms appear as masses of mycelia which are stained deep blue.

HAEMATOXYLIN - PICRO FUCHSIN

For nuclei, connective tissue, etc.

Solutions required:

A.	Distilled water	47·5 ml.
	Ferric chloride, hydrated 4% aqueous	2 ml.
	Hamaetoxylin 10% in absolute alcohol	0·4 ml.
B.	Picric acid, saturated, aqueous ..	20 ml.
	Acid fuchsin 1% aqueous	0·5 ml.
C.	Picric acid, saturated in absolute alcohol.	

Technique:

1. Tissues are fixed in Bouin and embedded in paraffin wax.

2. Sections about 8μ in thickness are fixed to slides, dewaxed with xylol and taken through the usual descending grades of alcohol to distilled water.

3. Stain for two to three minutes in solution A.

4. Differentiate and counterstain for about ten to fifteen seconds in solution B, controlling under the microscope, until only the nuclei are stained a greyish colour with the haematoxylin.

5. Rinse immediately in distilled water.

6. Dehydrate by dripping solution C onto the slide.

7. Clear with Terpineal.

8. Mount directly with Michrome mountant, or rinse with xylol, then mount with Clearmount or Cristalite.

Results:

Chromatin, black to grey. Muscle, yellow. Connective tissue, red. Keratinized regions, bright yellow. Cytoplasm, yellow.

Reference: Margolena, L. A. and Dolnick, E. H. (1951), *Stain Tech.*, vol. 26, pp. 119–21.

HAEMATOXYLIN - PICRO PONCEAU S

A selective stain for collagen and connective tissue in place of Haematoxylin - Van Gieson

Solutions required:

A. Haematoxylin (Heidenhain or Ehrlich).

B. *Picro Ponceau S (Curtis):*

Ponceau S 1% aqueous	10 ml.
Picric acid 1% aqueous	86 ml.
Acetic acid 1% aqueous	4 ml.

Technique:

Proceed exactly as for Haematoxylin (Ehrlich) - Van Gieson or Haematoxylin (Heidenhain) - Van Gieson (pages 127–128).

Results:

Identical with Haematoxylin - Van Gieson.

Note: Unlike Van Gieson, Picro Ponceau does not fade when mounted in Canada balsam: but Van Gieson does not fade when mounted in D.P.X. or Clearmount or Cristalite.

HAEMATOXYLIN (Weigert) - PONCEAU FUCHSIN (Curtis)

***Note:* The chief advantages of this method over numerous other trichrome techniques, is its simplicity and reliability and the fact that it works well after any fixative. The disadvantage is that cytoplasmic details are not as clearly revealed as after such stains as Masson or Mallory.**

Solutions required:

A. Weigert's Haematoxylin, A

B. Weigert's Haematoxylin, B

C. *Curtis Stain:*

Ponceau S, 2% aqueous	5 ml.
Picric acid, saturated, aqueous ..	95 ml.
Acetic acid 2%	2 ml.

Technique:

1. Fix material with any desired fixative, embed and section as usual.

2. Fix sections to slides and take down to 70% alcohol as usual.

3. If the fixative contains a salt of mercury, remove mercurial precipitate in the usual way.

4. Wash with distilled water.

5. Stain for five to ten minutes in a mixture consisting of one volume of each of solutions A and B, and two volumes distilled water.

6. Wash for five minutes in running water.

7. Stain for two to four minutes in Curtis Stain.

8. Wash with 95% alcohol.

9. Dehydrate with absolute alcohol.

10. Clear in xylol and mount.

Results:

Chromatin, black. Cytoplasm, yellow. Collagen and fibres, red.

Reference: Leach, E. H. (1946), *Stain Tech.*, **21**, 107–10.

HAEMATOXYLIN - PONCEAU FUCHSIN - FAST GREEN FCF

A micro-anatomical stain, superior to haematoxylin and eosin for the differentiation of tissues in histo-pathological work, superior to Van Gieson for collagen fibres. The stain has also been found very valuable in cytological work

Solutions required:

A. Iron haematoxylin (Heidenhain) No. 1.

B. Iron haematoxylin (Heidenhain) No. 2.

C. Picric acid 8% in 96% alcohol.

D. Acid fuchsin 1% aqueous .. 100 ml.
Glacial acetic acid .. .. 1 ml.

E. Ponceau de xylidine .. .. 1 gm.
Distilled water .. .. .. 100 ml.
Glacial acetic acid .. .. 1 ml.

F. Phosphomolybdic acid 1% aqueous.

G. Fast Green FCF 2% aqueous .. 100 ml.
Glacial acetic acid .. .. 2 ml.

Technique:

1. Bouin, formalin or Susa-fixed tissues are embedded and sectioned in the usual manner.

2. Immerse sections, which have been brought down to distilled water as usual, for one half to one hour in iron haematoxylin (Heidenhain) No. 1.

3. Rinse well in distilled water.

4. Stain in iron haematoxylin (Heidenhain) No. 2 for a length of time equal to the duration of stage 2 (above); then rinse thoroughly in distilled water.

5. Differentiate in solution C until only the nuclei are stained; then wash well in water; and stain in solution D for five minutes: wash in distilled water.

6. Stain one to five minutes in a mixture consisting of one part of Solution E (above) and nine parts of distilled water; then rinse thoroughly in tap water.

7. Differentiate in 1% phosphomolybdic acid for five to fifteen minutes until collagen fibres are almost colourless; then, without rinsing:

8. Stain in the Fast Green FCF solution for half to two minutes.

9. Differentiate by washing in water.

10. Dehydrate in the usual manner; clear and mount.

Results:

Nuclei are stained mauve to black; cytoplasm, varying shades of red and mauve; muscle, red; collagen fibres and mucus, green.

HAEMATOXYLIN - PONCEAU S - PICRO ANILINE BLUE

For differential staining of connective tissue and muscle

Solutions required:

A. Haematoxylin (Weigert) A.

B. Haematoxylin (Weigert) B.

C. Ponceau, S 0·2% aqueous .. 100 ml.
Glacial acetic acid .. .. 1 ml.

D. Picric acid, saturated aqueous .. 100 ml.
Aniline blue, water soluble .. 0·1 gm.

E. Acetic acid 1% aqueous.

Technique:

1. Tissues should be fixed in 10% formalin and paraffin sections employed.

2. Stain for five minutes in a freshly prepared mixture consisting of equal parts of Weigert's Haematoxylin A and B.

3. Wash in tap water.

4. Stain for three to five minutes in the acetic Ponceau, S (Solution C).

5. Rinse in distilled water.

6. Stain for three to five minutes in the picro aniline blue (Solution D).

7. Wash for three or four minutes in 1% acetic acid solution.

8. Dehydrate in ascending strengths of alcohol and clear in xylol in the usual manner.

9. Mount in acid balsam.

Results:

Connective tissue, glomerular basement membrane and reticulum, blue. Muscle and plasma, pink. Erythrocytes, bright red.

HAEMATOXYLIN (Weigert) SCARLET R

For demonstrating fatty acids crystals, soaps and neutral fats in fat necrosis

Solutions required:

A. Formalin 10% saturated with calcium salicylate.

B. Copper acetate 10% aqueous.

C. Weigert haematoxylin, A.

D. Weigert haematoxylin, B.

E. Borax 0·2% aqueous .. .. 1 litre
Potassium ferricyanide .. .. 2·5 gm.

F. *Scarlet R* (*Herzheimer*).
Acetone .. .. 50 ml.
Alcohol, 70% .. .. .. 50 ml.
Scarlet R .. .. 1·5 gm.
Heat on a hot water bath; then allow to cool before filtering.

Technique:

1. Fix tissues in Solution A; wash in running water and cut frozen sections.

2. Mordant the sections in Solution B for three to twenty-four hours; then wash in water.

3. Immerse in mixture of equal parts of Solutions C and D for twenty to forty-five minutes.

4. Differentiate in Solution E, examining under the microscope at intervals.

5. Wash well with distilled water.

6. Stain with Solution F for about five minutes.

7. Rinse quickly with 70% alcohol.

8. Rinse with distilled water.

9. Mount in neutral glycerine jelly.

Results:

Neutral fats are stained red, whilst fatty acids are deep blue black and haemoglobin, calcium and iron may also be stained.

Note: Calcium salicylate is added to the formalin fixative to convert soaps, which are sodium and potassium salts of fatty acids, into insoluble calcium soaps. If it is desired to demonstrate how much, if any, soap is present in addition to fatty acids, compare stained sections of two pieces of the same material, fixing one piece in Solution A and the other in ordinary 10% formalin.

HAEMATOXYLIN (Ehrlich) - VAN GIESON STAIN

A selective stain, for collagen and connective tissue, which requires less time than the Haematoxylin (Heidenhain) - Van Gieson technique, although the results are not as satisfactory

Solutions required:

A. Haematoxylin (Ehrlich).

B. Van Gieson stain (Picro Acid fuchsin).

Technique:

1. Mount sections on slides; dewax and pass through the usual descending grades of alcohol to water.

2. Stain in Ehrlich haematoxylin for ten to thirty minutes.

3. Rinse in water.

4. Blue in tap water or 1% lithium carbonate solution.

5. Stain for three to five minutes in Van Gieson stain.

6. Rinse for one or two seconds in water.

7. Drain and draw off excess water by applying a piece of filter paper to the edges of the section.

8. Dehydrate with absolute alcohol only.

9. Clear in xylol and mount in D.P.X., or Cristalite or Clearmount.

Note:

(*a*) It is essential, at stage 2, to overstain with haematoxylin as this stain is differentiated by the picric acid of the Van Gieson stain.

(*b*) Preparations mounted in Canada balsam fade, but fading can be obviated by use of one of the recommended mountants.

HAEMATOXYLIN (Heidenhain) - VAN GIESON STAIN

A selective stain for collagen and connective tissue, superior to Haematoxylin (Ehrlich) - Van Gieson

Solutions required:

A. Haematoxylin (Heidenhain) A.

B. Haematoxylin (Heidenhain) B.

C. Van Gieson stain.

Technique:

1. Fix pieces of tissue in Bouin, Carnoy, Susa or 10% formalin.

2. Fix sections to slides; dewax and take down to water in the usual way, after removing mercurial precipitate if Susa has been used as the fixative.

3. Immerse in solution A for one half to one hour.

Note: If a fixative other than Bouin, Carnoy, Susa or formalin has been used it will be necessary to increase the time in solution A and in solution B up to twelve hours or longer: the time varies for different fixatives.

4. Rinse in water.

5. Stain in solution B for a time exactly equal to step 3.

6. Rinse in water.

7. Differentiate with solution A, controlling by examination under the microscope, after the preparation has been rinsed briefly in water.

8. Wash gently in running water for about five minutes to remove all traces of solution A (iron alum).

9. Stain for three to five minutes in Van Gieson.

10. Rinse for a few seconds in water.

11. Examine, while still wet, under the microscope.

12. Continue the staining with Van Gieson, or continue the differentiation with water, whichever is necessary.

13. Drain and draw off excess water by means of a filter paper applied carefully to the edges of the section, but do not allow the preparation to dry completely.

14. Dehydrate with absolute alcohol only.

15. Clear in xylol.

16. Mount in D.P.X., Cristalite or Clearmount.

Results:

Nuclei of cells: dark brown to black. Collagen fibres: bright red. Erythrocytes, muscle, epithelia and other tissues: yellow.

Note: Van Gieson stain fades if mounted in Canada balsam, but fading can be avoided by the use of D.P.X., or Cristalite or Clearmount.

HICKSON'S PURPLE

A general stain suitable for class work

Solution required:

Hickson purple, saturated aqueous.

Technique:

1. Bring sections down to water as usual.
2. Stain in Hickson's purple for ten to twenty minutes.
3. Dehydrate, clear and mount.

Results:

Leucocytes, purple; erythrocytes, distinct red. The rest of the tissues purple.

Reference: Cannon, H. G. (1941), *J. Roy. Micr. Soc.*, series III, **61**, parts 3 and 4.

HICKSON'S PURPLE - VICTORIA GREEN

A general stain, particularly suitable for class work

Solutions required:

A. Hickson's Purple saturated aqueous.

B. Victoria green, G. saturated in 70% alcohol.

Technique:

1. Fix sections to slides; dewax and take through the alcohols down to distilled water as usual.
2. Stain for ten minutes in the Hickson's purple.
3. Rinse in distilled water.
4. Stain in the victoria green for half to one hour.

5. Rinse in 70%, followed by 90% alcohol.

6. Clear in xylol and mount.

Results:

Nuclear are sharply defined, purple. Erythrocytes sharply defined, stained vivid green, against a general blue-purple background.

Note: This method is an improvement of Hickson purple used alone.

Reference: Cannon, H. G. (1941), *J. Roy. Micr. Soc.*, series III, 61, parts 3 and 4.

HITCHCOCK AND EHRLICH'S MIXTURE

For lymphatic, ganglion, plasma and basophilic cells; immature cells of bone marrow, striated muscle, and fibrin.

Technique:

1. Fix in Zenker-acetic acid, corrosive sublimate, but not in Müller or formalin.

2. Paraffin sections are brought down to 90% alcohol; then passed through a solution of iodine in 90% alcohol.

3. The iodine is removed by passing through the graded alcohols to water and finally washing for fifteen minutes in running water.

4. Flood with the stain and allow it to act for fifteen to thirty seconds; then pour off the stain and wash rapidly in water.

5. The preparation is then passed directly into absolute alcohol, where it is allowed to remain only as long as the stain continues to be washed out in clouds.

6. Clear in xylol and mount.

Note: Sometimes the brilliancy of the stain is enhanced by re-staining.

Results:

Plasma cells: cytoplasm, brilliant crimson: nuclei, bluish-green. Other cells appear in lighter shades of green and crimson.

JENNER STAIN

For blood-forming organs

Solutions required:

A. *Formol-Saline.*

Formalin, conc. (i.e. 40% formaldehyde)	100 ml.
Sodium chloride, A.R.	8·5 gm.
Distilled water	1 litre
Acid sodium phosphate, monohydrate, A.R.	4 gm.
Anhydrous disodium phosphate, A.R.	6·5 gm.

B. Jenner stain.

Technique:

1. Fix pieces of tissue for two or three days in Solution A.

2. Dehydrate in ascending grades of alcohol as usual; clear; embed in paraffin wax.

3. Fix sections, not exceeding 5μ in thickness, to slides; de-wax; pass through the usual descending grades of alcohol down to distilled water which has been buffered to pH 7·0.

4. Stain for forty-five minutes in a grooved, stoppered staining jar, with a mixture consisting of equal volumes of Jenner stain and distilled water, buffered to pH 7.0.

5. Differentiate and dehydrate with absolute alcohol.

6. Clear in xylol and mount in Cristalite.

Results:

Neutrophile granules are stained pink. Oxyphile granules, brownish red. Basophile granules, purple. Nucleoli (plasmosomes), pink. The cytoplasm of partially haemoglobinated precursors of erythrocytes are stained in varying shades of reddish violet, while mature erythrocytes are deep pinkish orange.

JENNER STAIN - GIEMSA STAIN

For the polychromatic staining of blood-forming organs

Solutions required:

A. Formol saline.

B. Jenner Stain.

C. Giemsa stain .. .. .. 1 ml.
Distilled water (buffered to pH 7·0) 20 ml.

D. Acetic acid 0·08% aqueous

Technique:

1. Fix material in Solution A (above) from twelve to forty-eight hours.

2. Dehydrate in the alcohols and clear as usual; embed in paraffin wax and cut sections not exceeding 5μ in thickness.

3. Fix sections to slides and remove wax with xylol.

4. Wash well with two changes of pure methyl alcohol.

5. Stain sections with a measured volume of Jenner stain, which should be freshly filtered.

6. Cover the slides with a Petri dish lid lined with two or three sheets of moistened filter paper (this is to prevent the evaporation of the alcohol and the consequent formation of a precipitate on the sections), and allow the stain to act for three minutes.

7. Add a volume of distilled water (buffered to pH 7·0), equal to that of the stain, to the slides, which should now be gently rocked to ensure thorough mixing of the stain and water.

8. Allow this diluted stain to act for one minute.

9. Pour off excess stain; then without washing, immerse the slides in a stoppered staining jar containing diluted Giemsa stain (Solution C above) and leave the stain to act for forty-five minutes.

10. Rinse and differentiate in Solution D.

11. Rinse thoroughly in distilled water.

12. Dehydrate quickly in 95% alcohol, followed by two changes of absolute alcohol.

13. Clear in xylol and mount in Cristalite.

Results:

Erythrocytes are stained orange. Cytoplasm of lymphocytes and blastocytes are blue. Nuclei, deep blue to violet. Mast cell granules, violet to violet-red.

LEAD HAEMATOXYLIN - ACID FUCHSIN (MacConaill)

A " definitive " polychrome stain for the central nervous system and the trunks outside it.

The essence of this technique, which is due to Professor M. A. MacConaill, of the Department of Anatomy, University College, Cork, Ireland, is that the lead haematoxylin reduces the ammonium molybdate to form a blue lake, which makes it possible to employ only the minimum exposure to haematoxylin, thereby leaving the erythrophile (" Fuchsinophile ") parts of the neurone red.

Solutions required:

A.
Lead nitrate	2 gm.
Glacial acetic acid	8 ml.
Acid fuchsin	0·5 gm.
Water	92 ml.

B.
Haematoxylin	1 gm.
Acetic acid 4% aqueous	100 ml.

C.
Solution A	1 volume
Solution B	1 volume

Note: This solution should be prepared as and when required: it deteriorates after one day.

D.
Liquor ammon. acetat. B.P.	10 ml.
Ammonium molybdate, saturated, aqueous	70 ml.
Water	80 ml.

Note: All the above solutions must be made without the application of heat. Tap water may be used: the solutions must be filtered.

Technique:

1. Material should be fixed in 5 or 10% formalin and embedded in paraffin wax. Sections are cut 6 to 12μ in thickness.

2. Fix sections to slides; remove paraffin wax and take down to 70% alcohol by the usual stages.

3. Pass through 30% alcohol; then stain in Solution C for five minutes.

4. Rinse in two changes of tap water.

5. Immerse in Solution D for one to two minutes.

6. Wash in running water for two to five minutes to remove the unchanged molybdate.

7. Dehydrate; clear in xylol and mount.

Note: A deep yellow filter is of great help in microscopic examination, although not necessary.

Results:

Nuclei, dark blue; nucleoli of neurones, red; axial substances of nerve fibres, dark to pale blue; cuticular substance (including myelotheca) of nerve fibres, red; neurilemma (of Glees), purplish red.

Note: To eliminate all myelin, sections should be passed through Cellosolve after the alcohols. The same precaution should be observed when preparing tissue for embedding.

From *Proceedings of the Royal Irish Academy*, Vol. 53, Section B, No. 1, *The Myelothecal Apparatus of Human Nerve*; and from personal communications with Professor M. A. MacConaill, M.R.I.A.

LEISHMAN STAIN

For general differentiation of blood corpuscles; for malarial parasites; trypanosomes, etc.

This stain is extensively used by British workers who generally prefer it to Wright's stain which is used extensively in America.

Solutions required:

A. Formol saline, neutral, buffered.

B. Leishman stain.

C. Acetic acid 0·08% aqueous.

Technique:

1. Fix pieces of tissue in Solution A for sixteen to forty-eight hours.

2. Dehydrate in the usual ascending grades of alcohol; clear; and embed in paraffin wax.

3. Fix sections, not exceeding 5μ in thickness to slides; remove wax with xylol; pass through descending grades of alcohol down to neutral distilled water.

4. Stain for five to ten minutes in freshly prepared mixture consisting of one volume of Wright's stain and two volumes of neutral distilled water, in a stoppered staining jar.

5. Rinse with neutral distilled water.

6. Differentiate with the acetic acid solution, controlling by examination under the microscope, until the protoplasm of the cells is pink, and only nuclei are blue.

7. Wash with neutral distilled water.

8. Dehydrate quickly with absolute alcohol; clear in xylol; mount in Cristalite.

Results:

Erythrocytes, yellowish red. Polymorphonuclears: dark purple nuclei, reddish violet granules, pale pink cytoplasm. Eosinophiles: blue nuclei, red to orange-red granules, blue cytoplasm. Basophiles: purple to dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky blue cytoplasm. Platelets: violet to purple granules. Malarial parasites and Leishmania: chromatin, red; cytoplasm, blue. Trypanosomes: chromatin, red.

Note: The timing of the staining either before or after dilution may be altered to suit individual requirements. Staining effects similar to Giemsa are obtained by staining for ten minutes in Leishman stain diluted with twice its volume of distilled water buffered to pH 6·5.

LEUCO PATENT BLUE

For the identification of haemoglobin.

Solutions required:

A.	Patent blue AF54 (Michrome) ..	1 gm.
	Distilled water	100 ml.

Dissolve; then add:

Zinc metal powder	.. ..	10 gm.
Glacial acetic acid	.. ..	2 ml.

Boil until the blue colour completely disappears. Allow to cool; shake with about 1 gm. of decolorizing carbon; then filter. The liquid which should then be quite colourless is stored in a well stoppered bottle.

B. Solution B	.. .. ..	10 ml.
Glacial acetic acid	.. ..	2 ml.
Hydrogen peroxide 3%	..	1 ml.

N.B.: This solution must be freshly prepared and filtered before use.

C. Safranin 0·1% aqueous	..	99 ml.
Glacial acetic acid	.. ..	1 ml.

Technique:

1. Fix tissue blocks, not more than 3 to 5 mm. in thickness in 10% formalin buffered to pH 7·0 for 24 to 28 hours (prolonged fixation should be avoided).

2. Embed in paraffin wax as usual and cut section 5 to 6μ in thickness.

3. Take sections down to water as usual.

4. Stain in solution B for three to five minutes.

5. Wash briefly in water.

6. Counterstain for thirty to sixty sections in the safranin solution.

7. Rinse briefly with water.

8. Dehydrate as usual.

9. Clear in xylol.

10. Mount in Clarite or other synthetic mountant such as D.P.X., Clearmount, etc.

Results:

Haemoglobin, dark blue-green; background light pink.

Note: Blood and tissue smears fixed with methyl alcohol may also be stained by applying the stains as prescribed above.

Reference: Adapted from Dunn, R. C. (1946), *Stain. Tech.*, **21**, 65.

LEVADITI'S STAIN

For Treponema pallidum in sections

Solutions required:

A. Silver nitrate .. .. .. 2·5% aqueous

B. *Reducing solution:*
Pyrogallic acid .. .. .. 3 gm.
Formalin .. .. 5 ml.
Distilled water .. .. .. 100 ml.

Technique:

Tissues about 1 mm. thick should be fixed for twenty-four hours in 10% formalin and embedded in paraffin wax *after staining.*

1. After rinsing tissues in tap water, immerse in 95% alcohol for twenty-four hours.

2. Immerse in distilled water until the tissue sinks to the bottom of the jar.

3. Transfer to Solution A for 3 to 6 days at 37° C. in the dark, changing the solution every twenty-four hours.

4. Wash in distilled water; then immerse in Solution B for twenty-four to seventy-two hours in the darkroom at room temperature.

5. Wash in distilled water; then dehydrate with 80%, 95%, and absolute, alcohol.

6. Clear in cedarwood oil and embed in paraffin wax.

7. Sections are cut 5μ in thickness and mounted after removal of the paraffin wax.

Results:

Treponema, jet black. Tissue, yellow to brown.

LIGHT GREEN - ACID FUCHSIN (Alzheimer)

For demonstrating neuroglia changes

Solutions required:

A. Osmic acid 2% aqueous .. 20 ml.
Chromic acid 1% aqueous .. 75 ml.
Glacial acetic acid .. .. 0·5 ml.

B. Acid fuchsin 25% aqueous.

C. Picric acid, saturated, alcoholic 15 ml.
Distilled water .. .. .. 30 ml.

D. Light green 10% aqueous.

Technique:

1. Fix thin slices of the material in 10% formalin for twenty-four hours to three days.

2. Wash for twenty-four hours in running water.

3. Immerse very thin slices of the material in a comparatively large volume of Solution A which should be changed once or twice if it blackens.

4. Wash for several hours in running water.

5. Pass through ascending grades of alcohol.

6. Clear in the usual manner and embed in paraffin wax.

7. Sections not more than 2 to 4μ in thickness are fixed to slides.

8. De-wax with xylol.

9. Rinse thoroughly with absolute alcohol and pass through the usual descending grades of alcohol down to distilled water.

10. Stain for an hour at 60° C. with the acid fuchsin solution.

11. Allow the preparation to cool to room temperature; then wash with water.

12. Immerse in Solution C (picric acid) from one second to two minutes.

13. Rinse in two changes of water.

14. Stain from one half to one hour in the Light Green solution.

15. Rinse quickly in absolute alcohol.

16. Rinse in xylol.

17. Mount in Canada balsam or Cristalite.

Results:

Migrating astrocytes, of varying shades of green and sometimes containing fuchsinophile granules of brown stained lipoid inclusions. Lipoid contents of perivascular phagocytes are brown to

black. Neuroglia fibres and erythrocytes, red. Medullary sheaths are unstained. Connective tissue, deep green. Nerve cells are pale green with red stippling, while nerve-cell nuclei are a darker green with bright red nuclei.

Notes:

(*a*) The material must be fresh and only small pieces should be employed.

(*b*) Sections stained by this technique should appear lilac in colour to the naked eye.

(*c*) It is advantageous to experiment in order to determine the optimum staining time in the picric acid and the light green, as results vary according to the material to be stained.

From *Pathological Technique* by F. B. Mallory, by courtesy of Messrs. W. B. Saunders Co., Philadelphia, U.S.A

LIGNIN PINK

For whole mounts of marine invertebrates, particularly for crustaceans limbs, ostracod appendages, Medusa of Obelia, etc., as well as for demonstrating chitin

Overstaining with lignin pink is impossible, and it will not wash out with alcohol.

Solutions required:

A. *Sea water Bouin:*

Sea water saturated with picric acid	75 ml.
Formaldehyde 40%	25 ml.
Glacial acetic acid	5 ml.

B. Lignin pink saturated in distilled water or in Benzyl alcohol.

Technique:

1. Specimens are fixed from eighteen to forty-eight hours, according to the material, in Solution A.

2. Wash out the fixative with 50% alcohol, followed by 70% alcohol until the yellow coloration, due to the picric acid, is completely extracted.

3. Wash in running water to remove the alcohol.

4. Immerse in solution B for fifteen minutes or longer.

Results:

With the aqueous solution of the stain

Medusa of Obelia and limbs of crustaceans are stained deep carmine colour. The finest structures of ostracod appendages, uniform pink, but a better effect can, however, be obtained by staining the specimen for a longer period (up to sixteen hours) with a solution of the dye in benzyl alcohol: the final result in this case is a definite purple for the exoskeleton, while the other tissues are carmine colour.

Reference: Cannon, H. G. (1941), *J. R. Mic. Soc.*, series III, **61**, parts 3 and 4.

LITHIUM SILVER (Laidlaw)

For staining skin and tumours

Solutions required:

A. Iodine 1% in absolute alcohol.
B. Sodium thiosulphate 5% aqueous.
C. Potassium permanganate 0·5% aqueous.
D. Oxalic acid 5% aqueous.
E. *Lithium silver:*

Dissolve 12 gm. silver nitrate in 20 ml. distilled water in a 500 ml. stoppered bottle; then add 230 ml. lithium carbonate, saturated, aqueous, and shake well. Transfer to a 250 ml. measuring cylinder; cover with a watch glass and allow to stand undisturbed until the precipitate formed measures about 70 ml. Pour off the clear liquid and transfer the precipitate to another vessel. Wash precipitate with three or four changes of distilled water, decanting after each washing so that the precipitate remaining measures 70 ml. Add a diluted ammonia solution (15 ml. strong ammonia solution, sp. gr. 0·880 diluted with 35 ml. distilled water) a little at a time until the fluid precipitate is almost clear. Filter through a Whatman No. 40 filter paper.

F. Formalin 1% in tap water.
G. Gold chloride (yellow) 0·5% in distilled water.

Technique:

1. Fix tissues in 10% formalin for three days.
2. Dehydrate, clear, embed in paraffin wax in the usual manner.

3. Fix sections to slides, de-wax and take down to water as usual.

4. Wash in running water for five minutes.

5. Immerse in the iodine solution for three minutes.

6. Pour off excess iodine and immerse in the sodium thiosulphate solution for three minutes.

7. Rinse in tap water; then immerse in the potassium permanganate solution for three minutes.

8. Rinse in tap water.

9. Immerse in the oxalic acid solution for five minutes.

10. Wash in running tap water for ten minutes.

11. Immerse in three changes of distilled water for three or four minutes in each.

12. Stain in an oven for five minutes with the lithium silver solution heated to 50° C.

13. Rinse the slide back and front with distilled water to remove all traces of excess lithium silver.

14. Immerse slide in a jar of 1% formalin.

15. Rinse both sides of the slide with distilled water to remove all traces of the formalin solution.

16. Immerse in the yellow gold chloride solution in a coplin staining jar for ten minutes.

17. Rinse both sides of the slide with distilled water to remove all traces of excess gold chloride.

18. Flood the slides with oxalic acid and allow this reagent to act for ten minutes.

19. Rinse in distilled water.

20. Flood the sections with the sodium thiosulphate solution changing the solution every time it becomes turbid over a period of ten minutes.

21. Wash well in running water; then drain.

22. Dehydrate in ascending grades of alcohol, clear in xylol and mount.

Results:

Collagen is stained a reddish purple, while reticulum appears as black threads.

LORRAIN - SMITH - DIETRICH STAIN

For lipoids

Solutions required:

A. Potass. dichromate 5% aqueous.

Haematoxylin (Kultschitzky)

B. Haematoxylin 10% in absolute alcohol (ripened three months or longer) .. .. .. 10 ml.
Acetic acid 2% aqueous .. 90 ml.

C. Potass. ferricyanide .. .. 2·5 gm.
Borax 2% aqueous .. .. 100 ml.

Technique:

Material is fixed in 10% formalin and frozen sections are employed.

1. Mordant sections twenty-four to forty-eight hours in Solution A at 37° C.; then wash thoroughly in distilled water.

2. Immerse in Solution B at 37° C. for four to five hours; then wash in distilled water.

3. Differentiate overnight in Solution C.

4. Wash thoroughly in distilled water; drain and mount in Aquamount or in Farrant.

Results:

Lipoid substances, blue-black.

LUGOL'S IODINE

For the identification of glycogen in tissues

Solution required:

Lugol's iodine.

Technique:

1. Thin slices of tissue are fixed in absolute alcohol; then dehydrated; cleared, and embedded in paraffin wax in the usual manner.

2. Float sections on slides with 70% alcohol and flatten by warming gently, on a warm surface (but not with a direct flame).

3. Remove excess 70% alcohol by blotting very carefully but thoroughly.

4. Treat with xylol; then with absolute alcohol.

5. Stain in Lugol's iodine solution for ten minutes; then pour off excess stain and carefully blot the preparation thoroughly dry.

6. Clear and differentiate with origanum oil, controlling by examination under the microscope.

7. Mount in origanum balsam.

Results:

Glycogen, reddish brown; tissue constituents, pale yellow.

LUXOL FAST BLUE - CRESYL FAST VIOLET

(Klüver and Barrera's Stain)

For the combined staining of cells and fibres in the nervous system, obviating the need for chromate treatment and haematoxylin

Solutions required:

A.	Luxol fast blue 0·1% in 95% alcohol	100 ml.
	Acetic acid 10% aqueous	0·5 ml.
B.	Lithium carbonate 1% aqueous ..	5 ml.
	Distilled water	95 ml.
C.	Cresyl fast violet, CNS 0·1 to 0·25% aqueous	120 ml.
	Acetic acid 1% aqueous	1 ml.
D.	Xylol	1 volume
	Terpinol	3 volumes

Technique:

Material should be fixed in 10% formalin.

Paraffin or frozen sections give somewhat better results than Celloidin. Affixed Celloidin give better results than loose Celloidin sections.

(a) Frozen sections

1. Cut sections 25μ in thickness and place them in distilled water.

2. Immerse in 70% alcohol for ten to fifteen minutes.

3. Stain from five to twenty-four, but preferably not less than sixteen hours, in the Luxol fast blue solution, in a stoppered jar in an oven at 40° C.

***Note:* For staining four sections of the brain stem of a monkey, for example, 20 to 25 ml. of the stain should be used and then discarded.**

4. Immerse in 95% alcohol and wash off the excess stain.

5. Wash in distilled water.

6. Immerse for two or three seconds, but no longer, in the lithium carbonate solution, as the first stage of differentiation.

7. Continue the differentiation in several changes of 70% alcohol until the grey and white matter can be distinguished, but taking care not to over-differentiate.

8. Wash in distilled water.

9. Immerse in the lithium carbonate solution for three to five seconds, but no longer.

10. Complete the differentiation by immersing in several changes of 70% alcohol, until the white matter is stained greenish-blue in sharp contrast with the colourless grey matter.

11. Wash thoroughly in distilled water.

12. Stain for one to two minutes in the cresyl fast violet solution, which should be warmed carefully and filtered before use.

13. Wash for two or three seconds in distilled water.

14. Differentiate in several changes of 95% alcohol until colour ceases to come away from the preparation and the alcohol is no longer tinted.

15. Clear in xylol-terpineol (Solution D).

16. Clear in xylol and mount.

(b) Paraffin sections

1. Cut sections 15 to 20μ in thickness and fix to slides.

2. Remove paraffin wax with xylol and pass through absolute alcohol.

3. Rinse with several changes of 95% alcohol.

4. Stain with the Luxol fast blue solution for five to twenty-four hours, but preferably not less than sixteen hours, at 57° C. in an oven, taking precautions to prevent the loss of alcohol through evaporation from the staining solution.

5. Proceed exactly as at Stage 4 in the technique given above for frozen sections, except at Stage 11 the cresyl violet should be allowed to act for six minutes.

(c) Celloidin sections (loose)

1. Cut sections 15 to 30μ in thickness and place them into 75% alcohol.

2. Stain from five to twenty-four hours, but preferably not less than sixteen, in the Luxol fast blue solution at 57° C. in an oven, taking precautions to prevent the loss of alcohol by evaporation from the staining solution.

2. Proceed exactly as at Stage 4 in the technique given for frozen sections, except at Stage 11 the staining time for the cresyl fast violet should be increased to three minutes.

(d) Celloidin sections (affixed)

1. Cut sections 15 to 30μ in thickness, keeping the microtome knife and tissue continually flooded with 75% alcohol.

2. Place sections on slides, which have previously been smeared with glycerine albumen.

3. If necessary flatten out the sections on the slides by rolling with a piece of glass tubing half an inch in diameter and about two inches in length, or a small glass phial will serve the purpose.

4. Drop on sufficient clove oil to cover the sections and leave the oil to act for five minutes.

5. Remove the clove oil with 95% alcohol.

6. Remove the Celloidin with absolute alcohol.

7. Wash with 95% alcohol.

8. Proceed exactly as at Stage 2 in the technique given for frozen sections, except at Stage 2 the staining time for cresyl fast violet should be increased to six minutes.

Results:

Myelinated fibres are sharply contrasted greenish-blue against the reddish-coloured Nissl cells. The technique shows the Nissl picture and differentiates between the three types of glia cells: Myelin sheaths, neurons and glia nuclei are well demonstrated. Differentiation is also obtained between the three layers of medium-sized and larger blood cells, and capillary endothelium as well as mesothelial lining of Arachnoid membrane are sharply outlined. The finer fibres of the molecular layer of the cerebral cortex can be most effectively demonstrated in paraffin sections. Bacteria and pigments in nerve cells are more clearly demonstrated with this technique than with the usual Nissl stains.

References:
Klüver, Heinrich and Barrera, Elizabeth (1953), *J. of Neuropath and Exp. Neurology*, **12**, no. 4, 400–3, " A Method for the combined staining of cells and fibres in the nervous system ".
Klüver, Heinrich and Barrera, Elizabeth (1954), *J. of Psychology*, **37**, 199–223, " On the use of Azoporphin derivatives (Phthalocyanines) in the staining of nervous tissue ".

Note: In the original paper it is stated that this cell-fibre stain has been employed for peripheral nerves as well as structures of the central nervous system in amphibians, birds and mammals (rat, guinea-pig, rabbit, cat, monkey, chimpanzee, gorilla and man), and that with suitable counterstains Luxol fast blue will give excellent preparations of cochlea, adrenals and numerous extraneural tissues.

MacCALLUM'S STAIN

For influenza bacilli and Gram-positive organisms in tissues

Solutions required:

A. *Goodpasture's stain:*

Alcohol 30%	100 ml.
Basic fuchsin	0·59 gm.
Aniline oil	1 ml.
Phenol crystals	1 gm.

B. Picric acid saturated aqueous.

C. Stirling's gentian violet.

D. Gram's iodine.

E. Equal volumes of xylol and aniline oil.

Technique:

Tissues should be fixed in Helly and embedded in paraffin wax.

1. Stain for ten to thirty seconds in Solution A; then wash in tap water.

2. Differentiate for a few seconds in formalin till the bright red colour changes to a clear deep pink; then wash with tap water.

3. Counterstain one to five minutes in Solution B until the section appears purplish yellow to the naked eye; then wash with tap water.

4. Differentiate in 95% alcohol until the section appears red; then wash in tap water.

5. Stain for about five minutes in Solution C; then wash in tap water.

6. Stain for one minute in Solution D; then, without washing, blot dry.

7. Treat in Solution E until no more colour comes out.

8. Pass through two changes of xylol; then mount.

Results:

Gram-positive organisms, blue. Gram-negative, red. Tissues, varying shades of red and purple.

MALLORY'S STAIN - HAEMATOXYLIN

For the differential staining of the pancreatic islets

Solutions required:

A.	Distilled water	100 ml.
	Sulphuric acid conc.	1 ml.
	Potassium Permanganate	1 gm.

B. Harris or Ehrlich haematoxylin.

C. Lithium carbonate, saturated aqueous.

D. Acid Fuchsin (Mallory) 0·5% aqueous.

E. Phosphomolybdic Aniline Blue - Orange G (Mallory).

Technique:

1. Mammalian pancreases are fixed in Bouin and afterwards washed in 80% alcohol.

2. Dehydrate, clear, and embed in paraffin wax.

3. Cut sections 4 to 5μ in thickness.

4. Fix sections to slides: remove wax with xylol and pass through the usual grades of alcohol down to distilled water.

5. Immerse in solution A for about one half to one minute, until the sections appear uniform reddish brown in colour.

6. Rinse in distilled water.

7. Stain with Ehrlich or Harris Haematoxylin for five to ten minutes.

8. Blue in lithium carbonate solution.

9. Wash well in tap water.

10. Wash with distilled water.

11. Stain in Mallory's Acid Fuchsin, controlling under microscope until the A cells are red, and the B cells are pink.

If overstained, wash out with distilled water until the above effects are obtained.

12. Stain in solution E for twenty minutes to twelve hours according to the condition of the pancreas and degree of differentiation in the first stain.

Results:

Nuclei are stained dark violet; nucleoli, red. Cytoplasm in A cells contains red granules. Cytoplasm of B cells contains blue granules. Cytoplasm in acinar cells varies from red to pale violet with deep violet ergastoplasm. Canalicular cells, blue-grey. Connective tissue blue. Erythrocytes red. Mucus, azure blue.

Reference: Isaac, J. P. and Aron, C. (1952), *Bull. Mics. Appl.* ser. 2, **2**, 99–102.

MALLORY STAIN - HAEMATOXYLIN

For differential staining of acidophils, basophils and chromophobes in mouse pituitary

Solutions required:

A. *Zenker - Formol:*
   Zenker's Fluid .. .. .. 95 ml.
   Formaldehyde .. .. .. 5 ml.
B. Formic acid 10% aqueous.
C. Iodine 0·5% in 70% alcohol.
D. Sodium thiosulphate 0·75% in 10% alcohol.
E. Harris haematoxylin.
F. Lithium carbonate satd. aqueous.
G. Acid fuchsin 0·5% aqueous.
H. Phosphomolybdic acid 1% aqueous.
I. Mallory's Aniline blue-orange G.
J. Carbol xylol.

Technique:

1. Pituitary gland together with bone to which it is attached is fixed in solution A for 4–8 hours, or overnight in a refrigerator.

2. Wash in running tap water for 8 to 10 hours.

3. Decalcify by immersing in 10% formic acid for 24 hours.

4. Wash in running tap water for 2 to 4 hours.

5. Dehydrate, clear and embed in paraffin wax 56 to 58° C. in the usual way.

6. Cut sections about 4μ in thickness and mount on slides with glycerine albumen.

7. Dry the slides thoroughly in an oven at 56° C. for one to two hours, or overnight at room temperature.

8. Remove wax with xylol; then wash with two changes of absolute alcohol.

9. Wash with 90% followed by 70% alcohol.

10. Immerse for half to two minutes in solution C (Iodine).

11. Wash well with water.

12. Immerse in solution D (thiosulphate) for half to two minutes or until the natural colour of the sections is restored.

13. Wash well with tap water.

14. Rinse in distilled water.

15. Stain in Harris haematoxylin for 2–3 minutes.

16. Rinse in tap water.

17. Blue in the lithium carbonate solution for 1 minute.

18. Rinse well in distilled water.

19. Stain in the acid fuchsin solution for 1–2 minutes.

20. Rinse quickly in tap water.

21. Immerse in the phosphomolybdic acid solution for 2–5 minutes.

22. Without washing, pass the slides directly into Mallory's Aniline blue - orange G and leave therein for 1–2 hours.

23. Differentiate with 95% alcohol, controlling by examination under the microscope until the blue granules are intense, but the fuchsinophil granules are clearly visible.

24. Rinse with absolute alcohol.

25. Rinse with carbol xylol.

26. Immerse in two changes of xylol for 10 minutes in each.

27. Mount in Clearmount of D.P.X.

Results:

The nuclei of all cells are stained dark blue black. Granules of basophils, blue. Acidophil granules, brilliant red. Non-granular cytoplasm of chromophobes, light grey. Erythrocytes, orange to red. Bone, intense blue.

Reference: Gude, William D. (1953), *Stain Tech.*, **28**, no. 3, 161–2.

MALLORY STAIN - HAEMATOXYLIN (Ehrlich)

For Negri bodies in sections of brain

Solutions required:

A. Ehrlich haematoxylin.

B. Orange G. .. .. 0·5 gm.
Phosphotungstic acid, saturated aqueous .. .. 100 ml.

C. Acid fuchsin .. .. .. 0·5 gm.
Phosphotungstic acid .. .. 0·5 gm.
Acetic acid 1% .. .. .. 100 ml.

D. Phosphotungstic acid .. .. 2 gm.
Phosphomolybdic acid .. .. 2 gm.
Picric acid, saturated, aqueous .. 70 ml.
Absolute alcohol .. .. .. 30 ml.

E. Aniline blue, aqueous .. .. 1 gm.
Distilled water .. .. .. 98 ml.
Glacial acetic acid .. .. .. 2 ml.

Technique:

1. Fix paraffin sections to slides; dewax and take down to distilled water in the usual way.

2. Stain in Ehrlich haematoxylin for five minutes.

3. Blue in tap water, or in lithium carbonate solution, for two minutes.

4. Rinse in distilled water.

5. Stain for one minute in the orange G solution.

6. Wash in tap water until only the erythrocytes are stained yellow.

7. Rinse in distilled water.

8. Stain for ten minutes in the acid fuchsin solution.

9. Rinse in distilled water.

10. Differentiate for five minutes in solution D.

11. Rinse in distilled water.

12. Rinse in 1% acetic acid.

13. Dehydrate, clear and mount.

Results:

Negri bodies, purplish red with blue granulations. Cytoplasm of neurones, bluish. Nucleoli, dark purple. Erythrocytes, yellow.

Reference: Zlotnik, I. (1953), *Nature*, **172**, no. 4386, 962–3.

MALLORY HEIDENHAIN STAIN (Jane E. Cason's modification)

A rapid one-step method for connective tissue

Solutions required:

Phosphotungstic acid crystals A.R.	1 gm.
Orange G.	2 gm.
Aniline blue, water soluble ..	1 gm.
Acid fuchsin	3 gm.
Distilled water	200 ml.

Technique:

1. Fix pieces of tissue in Zenker-formol for preference, although Bouin's fluid, formalin and alcohol have been used with success.

2. Embed in paraffin wax and cut sections 6μ in thickness.

3. Fix sections to slides and remove wax with xylol.

4. Pass through descending grades of alcohol and if Zenker-formol has been used as the fixative, treat with iodine and sodium thiosulphate as usual to remove mercurial precipitate.

5. Take down to tap water.

6. Immerse for five minutes in the staining solution.

7. Wash in running tap water for three to five minutes.

8. Dehydrate rapidly through the usual graded alcohols.

9. Clear in xylol and mount.

Results:

Appear to be the same as those listed by Mallory (1938), i.e. collageneous fibrils, intense blue. Ground-substance of cartilage and bone, mucus, amyloid, and certain other hyaline substances are stained in varying shades of blue. Nuclei, fibroglia, myoglia and neuroglia fibrils, nucleoli, axis cylinders and fibrin are stained red. Erythrocytes and myelin, yellow. Elastic fibrils are stained pale pink or yellow.

Abstract: Cason, Jane E., *Stain Technology* (1950), **25**, No. 4, 225–6.

MALLORY'S PHOSPHOTUNGSTIC ACID HAEMATOXYLIN

A general stain for vertebrate tissues

Solutions required:

Haematoxylin 10% in absolute alcohol (ripened for three months or longer)	1 ml.
Phosphotungstic acid	2 gm.
Distilled water	100 ml.

Note: If ripened haematoxylin solution is not available, the following artificially ripened stain should be used:

Haematoxylin (dry) 0·1 gm., phosphotungstic acid 2 gm., distilled water 100 ml., potassium permanganate 1% aqueous 1·77 ml.

Technique:

1. Fix in Zenker. Embed in paraffin wax.
2. Bring sections down to distilled water.
3. Treat with iodine to remove mercuric percipitate.
4. Remove iodine with 0·5% aqueous sodium hyposulphite.
5. Wash thoroughly in running water.
6. Immerse for five to ten minutes in 0·25% potass. permanganate; then wash in tap water.
7. Immerse for ten to twenty minutes in 5% oxalic acid; then wash thoroughly with tap water.

8. Stain twelve to twenty-four hours in haematoxylin solution, prepared as above.

9. Wash in tap water; dehydrate with 95% and absolute alcohol.

10. Clear in xylol and mount.

Results:

Nuclei, centrioles, achromatic spindles, fibroglia, myoglia, neuroglia fibrils, fibrin, contractile elements of striated muscle, blue. Collagen, reticulum, ground substances of cartilage and bone, yellowish to brownish red. Coarse elastic fibrils, faint purple.

MARSHALL RED - VICTORIA GREEN

A general stain, particularly suitable for class work

Solutions required:

A. Marshall red, saturated aqueous.

B. Victoria green saturated in 70% alcohol.

Technique:

1. Fix sections to slides; dewax and take down to distilled water in the usual manner.

2. Stain in the Marshall red solution for twenty minutes.

3. Rinse in distilled water.

4. Stain in the Victoria green solution for half an hour.

5. Rinse in 70% alcohol followed by 90% alcohol.

6. Dehydrate with absolute alcohol.

7. Clear in xylol and mount.

Results:

Myofibrils, sage green. Nuclei, bright carmine. The results vary somewhat, but the muscle fibres always appear greenish to greenish-grey, while the nuclei are red. White matter of spinal cord, yellowish-green. Cartilage, pink. Retina stands out well as the rods and cones appear bright bluish-green. Erythrocytes unstained.

Reference: Cannon, H. G. (1941), *J. Roy. Micro. Soc.*, series III, 61, parts 3 and 4.

MASSON'S TRICHROME STAIN

For connective tissues

Solutions required:

A. Iron alum 5% aqueous.

B. Regaud's haematoxylin solution.

C. Picric acid, saturated in 95% alcohol .. .. 20 ml.
Alcohol 95% .. .. .. 10 ml.

D. Ponceau fuchsin.

E. Phosphomolybdic acid 1% aqueous.

F. Aniline Blue 5% in 2% acetic acid.

Technique:

1. Fix pieces of tissue in Bouin's fluid for three days or in Regaud's fluid for one day.

2. Wash in running water; dehydrate; clear and embed in paraffin wax as usual.

3. Sections 5μ in thickness are fixed to slides; de-waxed and passed through descending grades of alcohol down to distilled water in the usual manner.

4. Mordant in Solution A for five minutes at 45° C. to 50° C.

5. Wash well in distilled water.

6. Stain for five minutes in Regaud's haematoxylin at 45° C. to 50° C.

7. Rinse in distilled water.

8. Differentiate in picric alcohol (Solution C above) controlling by examination under the microscope, while the preparation is still wet.

9. Wash in running tap water for a minute or so.

10. Stain for five minutes in the Ponceau fuchsin solution.

11. Rinse in distilled water.

12. Differentiate in the phosphomolybdic acid solution for five minutes.

13. Add 0·5 ml. of the acetic aniline blue (Solution F above) to the phosphomolybdic acid on the slide and mix by rocking the slide gently. Allow this mixture to act for five minutes.

14. Pour off excess liquid and rinse in distilled water.

15. Immerse in phosphomolybdic acid solution again, for five minutes.

16. Transfer to 1% acetic acid and leave therein for five minutes.

17. Wash in distilled water.

18. Dehydrate in 95% alcohol, followed by absolute alcohol; clear in xylol; mount.

Results:

Collagen, deep blue. Neuroglia fibrils, red. Nuclei, black. Argentaffin granules, black or red.

METHYL BLUE - EOSIN (Mann)

For demonstrating the various types of cells in the anterior lobe of the pituitary and for the study of the relationship and development of the blood vessels

Solution required:

Methyl Blue-Eosin (Mann's stain).

Technique:

1. Paraffin sections of tissues which have been fixed in a fluid containing mercuric chloride are mounted on slides and treated by the standard technique for the removal of mercuric precipitate (*see* page 28).

2. Bring the sections down to distilled water; then stain for a quarter of an hour to two hours (the longer time is required if it is desired to demonstrate anterior lobe of pituitary).

3. Wash and differentiate in tap water.

4. Dehydrate rapidly with two changes of absolute alcohol.

5. Clear in xylol; mount in xylol balsam and examine under the low power, as the stain is too diffuse for critical high power work.

Results:

Nuclei are stained blue; karyosomes, dark blue; plasmosomes, red; basophil cytoplasm, blue; oxyphil cytoplasm and oxyphil granules, red.

METHYL GREEN

For amyloid

Solution required:

Methyl Green 1% aqueous.

Technique:

1. Fix tissues in absolute alcohol or in 10% formalin and cut frozen sections.
2. Immerse in the methyl green solution for five to ten hours.
3. Wash well in distilled water.
4. Mount in neutral glycerine, or in aquamount.

Results:

Amyloid is stained reddish violet, while other tissue elements are green.

METHYL GREEN - PYRONIN (Pappenheim-Unna)

For plasma cells

Solution required:

Methyl Green - Pyronin (Pappenheim-Unna).

Technique:

1. Paraffin sections of absolute alcohol-fixed tissues are mounted on slides and brought down to distilled water by the standard method.
2. Stain in Methyl Green - Pyronin (Pappenheim-Unna) from five to thirty minutes.
3. Rinse in distilled water; drain and blot carefully.
4. Dehydrate rapidly in two changes of absolute alcohol.
5. Clear in xylol; and mount.

Results:

Plasmosomes and oxyphil constituents of cytoplasm are stained red; chromatin reticulum and karyosomes, bluish green; cytoplasm of plasma cells, brilliant red; ground-substance of hyaline cartilage, yellowish red; bacteria, vivid red.

METHYL VIOLET 6B

For amyloid

Solution required:

Methyl Violet 6B aqueous 1%.

Technique:

1. Material should be fixed in alcohol and embedded in paraffin wax (although frozen sections may be employed).
2. Paraffin sections are fixed to slides and brought down to distilled water.
3. Stain for two to five minutes in the methyl violet 6B solution.
4. Rinse quickly in distilled water.
5. Differentiate with 1% acetic acid, controlling by examination under the microscope, until the amyloid appears reddish in colour.
6. Wash in distilled water.
7. Mount in glycerine jelly.

Results:

Amyloid substance is stained red, while cells and nuclei are in varying shades of blue.

Notes:

(*a*) The technique is not suitable for material which has undergone prolonged fixation in formalin.

(*b*) The stain is extracted from the amyloid by alcohol and for this reason glycerine jelly is used as the mountant, thereby obviating the use of alcohol for dehydration.

METHYL VIOLET - METANIL YELLOW

For typhus fever rickettsiae in lungs of mice

Solutions required:

A.	Methyl violet 10B	0·01% aqueous.
B.	Distilled water	90 ml.
	Acetic acid 1%	10 ml.
C.	Metanil yellow	0·01% aqueous.

Technique:

1. Fix pieces of lung in 10% neutral formalin.
2. Dehydrate, clear and embed in paraffin wax in the usual manner.
3. Fix sections to slides and take down to distilled water as usual.
4. Stain in the methyl violet solution for half to one hour.
5. Differentiate in solution B, controlling by examination under the microscope until the cytoplasm is decolorized.
6. Counterstain in the metanil yellow solution for a few seconds.
7. Dehydrate in acetone.
8. Mount in D.P.X. or Clearmount.

Results:

Rickettsiae are stained violet.

Reference: Nyka, W. J. (1945), *J. Path. and Bact.*, **52**, 317–24.

METHYL VIOLET - PYRONIN - ORANGE G (Bonney's Triple Stain)

For chromatin, connective tissue, keratin

Solutions required:

A.	Methyl violet 6B (Jensen) 1% aqueous	25 ml.
	Pyronin 10% aqueous	10 ml.
	Distilled water	65 ml.

B. Acetone .. .. 100 ml.
Orange G aqueous 2% .. .. About 10 ml.

Add orange G solution drop by drop to the acetone, with shaking, until the flocculent precipitate formed just redissolves with further addition of orange G solution.

Technique:

1. Small pieces of tissue are fixed in acetic-alcohol or in mercuric chloride.

2. Wash; dehydrate; clear; embed in paraffin wax.

3. If mercuric chloride has been used for fixation treat sections for the removal of mercuric precipitate by the standard method.

4. Take sections down to distilled water.

5. Immerse for two minutes in the methyl violet pyronin (Solution A above).

6. Pour off excess stain and carefully wipe the slide dry.

7. Flood the preparation with acetone-orange G solution.

8. Pour off after a few seconds.

9. Flood the preparation with a fresh lot of acetone-orange G solution and pour off after a few seconds.

10. Wash quickly in pure acetone.

11. Rinse with two lots of xylol.

12. Mount in balsam.

Results:

Cytoplasm, red; chromatin, violet; keratin, violet; connective tissue, yellow.

METHYLENE BLUE - BASIC FUCHSIN

Rapid method of demonstrating Negri bodies in sections

Solutions required:

A. Methylene blue .. .. .. 1 gm.
Basic fuchsin .. .. .. 1·75 gm.
Methyl alcohol, pure .. .. 100 ml.
Glycerine, pure .. .. .. 100 ml.

B. Potass. hydroxide N.20 .. .. 6·25 ml.
Distilled water .. .. .. 93·75 ml.

C. Solution A .. .. .. 10 ml.
Solution B .. .. .. 0·25 ml.

Note: Solution C should be freshly prepared as required.

Technique:

1. Blocks not exceeding 3 mm. in thickness of fresh tissue from the hippocampus major and cerebellum should be fixed in Zenker for twelve to twenty-four hours.

2. Remove mercurial precipitate with iodine in alcohol by the usual method (see page 28); then wash in running water for three to six hours.

3. Dehydrate in dioxane (see page 36) and embed in paraffin wax.

4. Sections not more than 4μ thick are mounted on slides and brought down to distilled water.

5. Flood slides with Solution C and steam gently for five minutes; then cool and wash rapidly in tap water.

6. Decolorize and differentiate each section separately by waving the slide gently in a jar of 90% alcohol until the section assumes a faint violet colour.

7. Dehydrate rapidly in 95% and absolute alcohol; clear in xylol and mount.

Results:

Negri bodies, deep red; granular inclusions, dark blue; nucleoli, bluish black; cytoplasm, bluish violet; erythrocytes, dull reddish brown.

METHYLENE BLUE - BASIC FUCHSIN

For rickettsia in sections

Solutions required:

A. Methylene blue 1% aqueous.

B. Basic fuchsin 0·5% aqueous.

C. Citric acid 0·5% aqueous.

Technique:

1. Tissues are fixed in Regaud's fluid, washed in running water, dehydrated, cleared and embedded in paraffin wax as usual.

2. Fix sections to slides; de-wax; pass through the alcohol down to distilled water in the usual manner.

3. Stain in the methylene blue solution for twelve to sixteen hours.

4. Decolorize with 95% alcohol.

5. Counterstain with the basic fuchsin solution for fifteen minutes.

6. Decolorize for one to three seconds in the citric acid solution.

7. Pour off excess citric acid; rinse in distilled water; drain and blot carefully.

8. Decolorize and dehydrate rapidly in absolute alcohol.

9. Clear in xylol and mount in dammar xylol.

Results:

Rickettsia are stained a deep red; surrounding tissue, pink; background, light blue.

METHYLENE BLUE POLYCHROME (Unna)

For mast cells in sections

Solutions required:

Methylene blue, polychrome (Unna)	100 ml.
Potash alum	5 gm.

Technique:

1. Material is fixed in absolute alcohol and embedded in Celloidin.

2. Stain sections in a watch glass from three to sixteen hours in the methylene blue solution.

3. Rinse well in distilled water.

4. Dehydrate with 95% alcohol.
5. Clear in origanum oil.
6. Mount in Canada balsm or in Cristalite.

Results:

Mast cell granules are stained red; nuclei, blue.

METHYLENE BLUE POLYCHROME - GLYCERINE ETHER (Unna)

For differentiating mast cells and plasma cells

Solutions required:

A. Formalin (40% formaldehyde) .. 50 ml.
Absolute alcohol .. .. 100 ml.

B. Polychrome methylene blue (Unna)

C. Glycerine ether (Unna) .. .. 5 ml.
Distilled water .. .. .. 35 ml.

Technique:

1. Fix tissues in Solution A; dehydrate; clear; embed in paraffin wax.

2. Immerse sections in the methylene blue solution for ten minutes.

3. Rinse in distilled water.

4. Differentiate in the glycerine ether mixture from one half to one minute until the sections appear to be deep sky blue to the naked eye (care should be taken that the sections are not over-differentiated).

5. Wash thoroughly in distilled water for a few minutes.

6. Fix sections to slides and carefully blot with filter paper.

7. Dehydrate rapidly with absolute alcohol.

8. Clear in xylol and mount.

Results:

Nuclei are stained blue, while mast cells are red and plasma are blue.

MUCICARMINE - METANIL YELLOW - HAEMATOXYLIN

For mucin and connective tissue

Solutions required:

A. Haematoxylin (Weigert) A.

B. Haematoxylin (Weigert) B.

C. Metanil yellow 0·25% aqueous.

D. Mucicarmine (Southgate).

Technique:

1. Fix material in 10% formalin.

2. Dehydrate, clear; embed in paraffin wax.

3. Fix sections to slides; de-wax with xylol and pass through the usual descending grades of alcohol.

4. Rinse with distilled water.

5. Stain sections for one minute in a freshly prepared mixture consisting of equal volumes of Solution A and B.

6. Wash in distilled water.

7. Immerse in Solution C for about two minutes.

8. Rinse quickly with distilled water.

9. Immerse in the mucicarmine solution for forty-five minutes.

10. Rinse quickly with distilled water.

11. Rinse quickly with 95% alcohol.

12. Dehydrate rapidly but thoroughly with absolute alcohol.

13. Clear in xylol and mount.

Results:

Mucin is stained red, while connective tissue is yellow, and nuclei are black.

MUCICARMINE (Mayer)

Solutions required:

Mucicarmine, stock solution ..	1 volume
Alcohol 70%	10 volumes

Note: This diluted solution should be freshly prepared.

Technique:

Tissues should be fixed in absolute alcohol for five to eight hours and embedded in paraffin wax, Celloidin or L.V.N.

1. Bring paraffin sections down to distilled water; then stain for ten to twenty-five minutes in the above solution.

2. Wash rapidly with distilled water.

3. Dehydrate with 70%, 95% and absolute alcohol.

4. Paraffin sections are cleared in xylol; Celloidin or L.V.N. sections are cleared in terpineol or origanum oil.

Results:

Mucin is stained red.

MUCICARMINE (Southgate)

This is used in accordance with the Mayer technique, but 1 volume of the stock solution is diluted with 9 volumes of distilled water instead of 10 volumes 70% alcohol.

Southgate's modification gives more uniform results than Mucicarmine prepared by Mayer's original formula.

MUCIHAEMATEIN

For mucus

Solution required:

Haematein	0·2 gm.
Aluminium chloride	0·1 gm.
Glycerine, pure	40 ml.
Distilled water	60 ml.

Technique:

1. Material is fixed in absolute alcohol; cleared; and embedded in paraffin wax.

2. Sections are stained for ten minutes in the above solution.

3. Wash with distilled water.

4. Dehydrate by plunging the slide into two or three changes of 95% alcohol.

5. Pass through absolute alcohol; then clear in xylol; mount.

Results:

Mucus is stained blue, while the remainder is colourless.

NADI REACTION

For oxidase granules

Solutions required:

A. a-Naphthol .. .. .. 1 gm.
Distilled water .. .. .. 100 ml.

Place in a 250-ml. flask and boil until the a-Naphthol begins to melt; then add 40% potassium hydroxide aqueous solution drop by drop until the solution becomes yellowish-blue in colour and the a-Naphthol is still not completely dissolved.

B. Cold tap water .. .. .. 100 ml.
*Dimethyl-p-phenylenediamine base .. .. 0·5 gm.

Place the water in a clean amber bottle, open the ampoule by filing a groove at one end then breaking in the usual manner. Tip the contents of the ampoule into the bottle; then replace the stopper and allow the bottle to stand, with occasional shaking, for twenty-four hours. Care should be taken that the Dimethyl-p-phenylenediamine base does not come into contact with the body.

This solution deteriorates after three or four weeks.

**Note:* This should be purchased in a sealed ampoule.

C. Gram's iodine solution.

D. Carmalum.

Technique:

1. Thin pieces of fresh tissue (not more than 3 mm. thick) are fixed for three to five hours in formalin-saline solution.

2. Make frozen sections and collect them in distilled water.

3. Immerse in a mixture consisting of equal volumes of Solutions A and B (*Note:* This mixture must be made and filtered immediately before use) for about five minutes until the sections turn blue.

4. Rinse sections rapidly in distilled water.

5. Immerse in Gram's iodine solution until the sections turn brown.

6. Transfer to distilled water to which two drops of lithium carbonate 0·5% aqueous have been added for each 100 ml. of distilled water, for a quarter to twenty-four hours until the sections have regained their blue colour.

7. Wash in distilled water then counterstain in carmalum for two to five minutes.

8. Mount in glycerine jelly or Apathy's medium or in Aquamount.

Results:

Oxidase granules are stained blue while nuclei are pink. Sometimes fat is stained also.

NAPHTHOL BLUE BLACK - HAEMATOXYLIN - BRILLIANT PURPURIN AZOFUCHSIN

For collagen, reticulum, smooth muscle, etc.

Solutions required:

A. Weigert's Haematoxylin A.

B. Weigert's Haematoxylin B.

C. Brilliant purpurin R. (C.I. No. 454) in 1% acetic acid .. .. .. 30 ml.
Azofuchsin 1% in 1% acetic acid.. 20 ml.

D. Naphthol blue black (C.I. No. 246) 1 gm.
Picric acid, satd., aqueous .. 100 ml.

Technique:

1. Paraffin sections are fixed to slides and taken down to 70% alcohol in the usual manner.

2. Stain for six minutes in a freshly prepared mixture consisting of equal parts of solutions of A and B.

3. Wash in tap water.

4. Counterstain for five minutes in solution C.

5. Wash in 1% acetic acid.

6. Stain in solution D for five minutes.

7. Rinse in 1% acetic acid for two minutes.

8. Pass through the usual ascending grades of alcohol dehydrate in absolute.

9. Clear in Xylol.

10. Mount in D.P.X. or Clearmount.

Results:

Collagen, reticulum and basement membranes, dark green. Smooth muscle, brown. Nuclei, brownish black.

Reference: Lillie, R. D. (1945), *J. Tech. Meth.*, **25**, 47.

NAPHTHOL GREEN B - HAEMATOXYLIN (Weigert)

For connective tissue

Solutions required:

A. Weigert's haematoxylin, A.

B. Weigert's haematoxylin, B.

C. Eosin, yellowish, 1% in tap water.

D. Ferric chloride, hydrated 10%.

E. Naphthol Green B, 1% aqueous.

F. Equal volumes of acetone and xylol.

Technique:

1. Paraffin sections are mounted on the slide and brought down to distilled water in the usual manner.

2. Stain for six minutes in a freshly prepared mixture consisting of equal volumes of Weigert's haematoxylin A and B.

3. Wash thoroughly in tap water; then stain for three minutes in the eosin solution.

4. Wash in tap water; then immerse in the ferric chloride solution for five minutes.

5. Rinse well in distilled water; then stain for five minutes in the naphthol green solution.

6. Differentiate for two or three minutes in 1% acetic acid.

7. Drain well; then dehydrate with acetone, afterwards clearing in acetone-xylol (as above); then mount.

Results:

Connective tissue, green; muscle and cytoplasm, pink.

Reference: Lillie, R. D. (1945), *J. Tech. Meth.*, 25, 32.

NEUTRAL RED - FAST GREEN

For staining both Gram-positive and Gram-negative bacteria in sections

Solutions required:

A. Aniline crystal violet.

B. Gram's iodine.

C. Absolute alcohol .. .. 98 ml.
Glacial acetic acid .. .. 2 ml.

D. Twort's stain, modified (neutral red–fast green)

Technique:

1. Fix tissue in 5% formal-saline, dehydrate, clear; embed in paraffin wax. Cut sections 3μ in thickness.

2. Stain in aniline crystal violet for three to five minutes.

3. Pour off excess and blot, without washing.

4. Flood with Gram's iodine and allow the stain to act for three minutes.

5. Destain with the acetic acid alcohol (Solution C above) until no more colour comes away, and the sections assume a dirty straw colour.

6. Rinse quickly in distilled water.

7. Stain in neutral red-fast green diluted one part with three parts of distilled water, for five minutes.

8. Wash quickly with distilled water.

9. Decolorize with the acetic alcohol solution until no more red stain comes out.

10. Rinse quickly in absolute alcohol.

11. Clear in xylol and mount.

Results:

Nuclei are stained red, while cytoplasm is light green. Gram-positive bacteria, dark blue. Gram-negative bacteria, pink. Erythrocytes, green.

J. Path. & Bact., 59, 357–8, Ollett, W. S. (1947).

NILE BLUE SULPHATE

For demonstrating fatty acids and neutral fats

Solution required:

To 100 ml. saturated aqueous Nile Blue sulphate add 0·5 ml. conc. sulphuric acid; then boil under a reflux condenser for two hours; allow to cool; then use as follows:

Technique:

1. Fix small pieces of tissue in 10% formalin.

2. Frozen sections are stained for about ten minutes to half an hour at 37° C.; or overnight at room temperature.

3. Differentiate in 2% acetic acid.

4. Rinse in distilled water; mount in Farrant.

Results:

Free fatty acids, blue. Neutral fats, red.

NILE BLUE - PICRO FUCHSIN (Murray-Drew)

For bacteria and actinomyces in pathological tissues

Solutions required:

A. Picro fuchsin (Van Gieson).

B. Nile Blue sulphate aqueous 1%.

Technique:

1. Formalin-fixed material is embedded in paraffin wax, or frozen sections may be employed.

2. Take sections down to distilled water in the usual manner then stain in picro fuchsin for two or three minutes.

3. Wash with distilled water.

4. Stain in Nile Blue sulphate for four to twenty-four hours.

5. Rinse in distilled water until the washings are tinted pale blue.

6. Drain off excess water; then blot the preparation carefully but thoroughly.

7. Dehydrate rapidly in absolute alcohol; then clear in xylol.

8. Differentiate in clove oil for five to fifteen minutes (for paraffin sections) or for several hours in the case of thick frozen sections.

9. Rinse in two or three changes of xylol to remove clove oil; then mount.

Results:

Bacteria and chromatin are stained blue; collagen fibres, red; mast cell granules, blue-black; fibrin, blue or reddish orange; erythrocytes and keratin, yellow.

ORANGE G - CRYSTAL VIOLET (Bensley)

For secretion antecedents of serous or zymogenic cells. This stain is particularly suitable for sections of the stomach or pancreas

Solutions required:

A. Osmic acid 2% .. .. .. 4 ml.
Potassium dichromate 5% .. 4 ml.
Glacial acetic acid .. .. 1 drop
Distilled water .. .. 2 ml.

B. *Neutral gentian*
Orange G 8% aqueous .. .. 25 ml.
Crystal violet 4% aqueous .. 25 ml.

Mix thoroughly by shaking until almost complete precipitation takes place; then allow the preparation to stand for an hour, afterwards collecting the precipitate on a filter. Wash the precipitate on the filter with about 250 ml. distilled water; then dry and dissolve it in 25 ml. absolute alcohol.

C. Solution B (as above) .. .. 10 ml.
Alcohol 20% .. .. Sufficient to impart a rich port-wine colour.

Allow to stand for twenty-four hours; then filter.

D. Absolute alcohol .. .. 1 volume
Clove oil .. .. 3 volumes

Technique:

1. Tissues are fixed in Solution A (as above) for twenty-four hours, and afterwards embedded in paraffin wax in the usual manner.

2. Stain sections for twenty-four hours in Solution C (as above); then pour off excess stain, and blot the preparation carefully.

3. Dehydrate by immersing in two or three changes of acetone.

4. Clear in xylol; then differentiate in Solution D (as above), controlling under the microscope.

5. Rinse in two changes of xylol; then mount in balsam.

Results:

Zymogen granules, violet; granules of acidophil cells are stained orange-red, while those of basophil cells are violet; background, brown.

ORCEIN

For elastic fibres and connective tissue

Solutions required:

A. Orcein .. .. 1 gm.
70% alcohol .. .. .. 100 ml.
Heat on a water bath to dissolve; cool; filter; then add:
Hydrochloric acid, conc. .. 1 ml.
Shake well.

B. Unna's polychrome methylene blue.
This should be diluted 1 : 10 or 1 : 15 for use.

1. Fix tissues (any fixative may be employed); dehydrate; clear; and embed.

2. Paraffin sections are brought down to 70% alcohol in the usual manner; flood with freshly filtered orcein solution, prepared as above, and warm gently for ten to fifteen minutes, until the solution thickens. Alternatively, the sections may be stained overnight at room temperature.

3. Wash thoroughly with 70% alcohol.

4. Wash thoroughly with distilled water.

5. Stain in diluted polychrome methylene blue until the nuclei are bright blue to blue-black; the time necessary may be ascertained by examining under the microscope at intervals.

6. Differentiate in 95% alcohol, followed by absolute.

7. Clear in xylol and mount.

Results:

Elastic fibres, deep brown. Nuclei, bright blue or blue to black. Connective tissue, pale brown.

Note: If it is desired only to stain the elastic fibres, omit No. 5, and treat sections with acid alcohol for a few seconds before washing with distilled water.

ORCEIN - ANILINE BLUE - ORANGE G

A differential stain for elastic fibres, collagen, keratin, etc.

Solutions required:

A. Orcein .. .. 1 gm.
Alcohol 70% .. .. 100 ml.
Hydrochloric acid, conc. .. .. 0·6 ml.

B. Alcohol 50% .. .. .. 49 ml.
Hydrochloric acid, conc. .. .. 0·5 ml.

C. *Mallory's Aniline Blue - Orange G.*
Aniline blue, aqueous .. .. 0·5 gm.
Orange G .. .. 2 gm.
Phosphomolybdic acid 1% .. .. 100 ml.

D. Orange G 1% in absolute alcohol.

Technique:

1. Fix material in Bouin and embed in paraffin wax.

2. Sections, about 8μ in thickness, are fixed to slides, dewaxed with xylol and passed through the usual descending grades of alcohol to distilled water.

3. Stain for one and a half hours in the orcein solution in a closed staining jar.

4. Differentiate with solution B, controlling under the microscope, until most of the pink is extracted from the sections.

Note: The duration of the differentiation will vary according to the nature of the material and to the thickness of the sections.

5. Wash thoroughly with running tap water.

6. Wash with distilled water.

7. Immerse in solution C diluted with an equal volume of distilled water, for one to two minutes.

8. Rinse with 95% alcohol.

9. Rinse with two lots of solution D.

10. Rinse quickly with absolute alcohol.

11. Clear in xylol and mount.

Results:

Elastic fibres, red. Collagen, blue. Muscle fibres, pale orange to dirty yellow. Cytoplasm, varying shades of yellow. Erythrocytes, golden yellow. Kerantinized material, bright yellow.

Reference: Margolena, L. A. and Dolnick, E. H., *Stain Tech.*, (1951), **26**, 119–21.

ORCEIN - ANILINE SAFRANIN

For elastic and connective tissue fibres

Solutions required:

A.	Orcein (Unna) ½ to 1% in 80% alcohol	100 ml.
	Hydrochloric acid, conc. ..	1 ml.
B.	Safranin O, water soluble ..	1 gm.
	Aniline water	48 ml.
	Absolute alcohol	52 ml.

Technique:

1. Sections are mounted on slides and brought down to 90% alcohol in the usual manner. (If the tissues have been fixed in a fluid containing mercury, the mercurial deposit is removed by the standard technique; the sections are then taken from 70% to 90% alcohol, then direct into the orcein stain.)

2. Stain from twenty to sixty minutes with the orcein solution in a stoppered staining jar.

3. Rinse in acid alcohol until stain ceases to come out of the preparation.

4. Rinse in 70% alcohol; then in distilled water.

5. Stain in aniline safranin (Solution B) for five minutes; then rinse in water.

6. Differentiate by dipping into 90% alcohol then examining rapidly under the microscope. Repeat this process until the cell nuclei are well brought out, stained clear bright red.

7. Dehydrate by rinsing quickly in absolute alcohol; then clear in xylol and mount in balsam.

Results:

Elastic fibres are stained dark to reddish brown; cell nuclei, bright red; ground substance of hyaline cartilage, yellow.

ORCEIN - GIEMSA STAIN

For syphilitic tissue, particularly dermatological specimens

Solutions required:

A. *Orcein (Unna-Tanzer):*

Orcein	0·5 gm.
Alcohol 70%	100 ml.
HCl, concentrated	0·6 ml.

B.

Absolute alcohol	100 ml.
HCl, concentrated	0·5 ml.

C.

Giemsa stain	0·25 ml.
Distilled water	100 ml.

Note: Solution C should be prepared freshly, as required, from Giemsa stain.

D.

Absolute alcohol	100 ml.
Eosin 1% alcoholic	1 ml.

Technique:

1. Paraffin sections are brought down to 70% alcohol.

2. Stain for one half to one hour in Solution A; then rinse for two to five minutes in distilled water.

3. Wipe off excess water; dip into 95% alcohol for a few seconds; then decolorize with absolute alcohol for five to twenty-

five minutes, or until the sections assume a pale brown colour and the elastic fibres stand out, deep purple to black, under the low-power objective.

4. Decolorize in Solution B until the background is almost colourless. This usually takes two to seven minutes.

Note: Decolorization must not be extended more than ten minutes, as otherwise the thin elastic fibres will become destained.

5. Immerse in tap water for five to ten minutes.

6. Stain for two to twelve hours with Solution C until the epithelial and other cells are deep blue; connective tissue, greyish pink or greyish blue or blue.

7. Wipe off excess stain and dehydrate and decolorize in Solution D, controlling under the microscope.

Note: Decolorization must be stopped when the connective tissue has lost all trace of blue and has assumed a rose tint. The blue tinge is removed fairly rapidly in Solution D. The epidermis should remain bright blue.

8. Immerse in two changes of absolute alcohol for two minutes in each. Clear in xylol, and mount.

Results:

Nuclei, deep blue. Cytoplasm of the epidermis, muscle cells and connective tissue cells, light blue. Plasma cells, dark greyish blue. Eosinophilic granules, bright red. Mast cell granules, metachromatic (varying shades of) purple. Neutrophilic granules, only faintly stained. Erythrocytes, reddish brown. Collagenous fibres, pale rose to brownish pink. Elastic fibres, dark brown to black. Senile degenerated connective tissue (collacin, elacin and collastin), various shades of dark grey and blue. Cartilage, metachromatic (varying shades of) purple. Decalcified bone, light brown. Keratin, blue (poorly stained). Stratum lucidum, dark red; keratin layer may be light blue or light pink or colourless depending upon the tissue and the degree of decolorization. Inner root sheath of the hair, deep blue. Melanin granules, green to black. Other pigments, unstained. Bacteria and mycelia, deep blue. Demodex folliculorum in hair follicles, brown with blue granulations.

ORCEIN - PICRO FUCHSIN (Van Gieson)

For elastic and collagen fibres

Solutions required:

A. Orcein (Unna) 1% in 80% alcohol 100 ml.
Hydrochloric acid, conc. .. 1 ml.

B. Picro-fuchsin (Van Gieson).

Technique:

1. Sections are mounted on slides and brought down to 70% alcohol in the usual manner. If tissues have been fixed in a fluid containing mercury, the mercurial precipitate is removed by the standard technique.

2. Immerse in orcein solution (formula as above) for half an hour or longer if necessary; then rinse in acid alcohol.

3. Rinse in 70% alcohol; then in water.

4. Stain with picro fuchsin (Van Gieson) for three to five minutes.

5. Rinse rapidly (not more than a few seconds) in water.

6. Dehydrate rapidly; clear, then mount.

Results:

Collagen fibres are stained red; elastic fibres, brown; erythrocytes, epithelia, muscle, etc., yellow.

OSMIC ACID

A rapid technique for staining fat in frozen sections

Solutions required:

A. Osmic acid 1% aqueous.
Note: Store in an amber bottle.

B. Eosin, yellowish, 1% aqueous.

Technique:

1. Tissues are fixed as follows:
Place 22·5 ml. distilled water in a 50-ml. beaker and heat to about 90° C.; then add 2·5 ml. formalin; raise to boiling point;

drop in a thin piece of the tissue; then place the beaker in an oven at 60 to 65° C. for ten minutes.

2. Cut frozen sections 10μ thick, and place them in another 50-ml. beaker.

3. Boil Solution A in a large test-tube and pour onto the sections, then transfer to an oven at 60° C. for five minutes.

4. Wash sections in a dish of cold tap water after pouring back the osmic acid, which may be used again, into the stock bottle.

5. Counterstain in Solution B for one minute.

6. Wash quickly in tap water; transfer sections to slides; drain and mount in glycerine jelly, previously melted in an oven or on a water bath.

Results:

Fat globules, black or greyish black against a red background.

CAUTION: Osmic acid vapour is injurious to the eyes.

PASINI'S STAIN (Improved)

For differentiation of connective tissue

Solutions required:

A. Iron alum 2·5% aqueous.

B. *Pasini's stain:*

Unna's aniline blue-orcein ..	10 ml.
Eosin bluish 2% in 50% alcohol..	12 ml.
Acid fuchsin	0·3 gm.
Neutral glycerine..	5 ml.

Technique:

1. Tissues should be fixed in Heidenhain's susa mixture and embedded in L.V.N. Sections are cut 3μ in thickness.

After removal of mercuric precipitate in the usual manner sections are mordanted in Solution A for twenty-four hours.

2. Transfer to Solution B for three to ten minutes.

3. Transfer to 95% alcohol and agitate for about one minute or until the colour ceases to come out in clouds.

4. Immerse in absolute alcohol for one minute; then blot, clear and mount.

Results:

Collagen fibres, deep blue. Cytoplasm, red. Epithelial cells, centroiles, basal bodies, nuclear structure, brilliant red. Erythrocytes, yellowish red. Connective tissue, blue. Secretory bodies, varying according to their nature. Slime of goblet cells, azure blue. Connective tissue, wandering cells, smooth and striated muscle, well defined.

PERIODIC ACID (HOTCHKISS) - CELESTIN BLUE

For human and animal pituitary glands, demonstrating both muco-protein precursors of the gonadotrophins

Compared with other methods, finer differences in the cytology of the cyanophils can be appreciated. Cell counts can readily be carried out, and the counts are more accurate, giving more definite and clearer results than those obtained by older methods: for instance cells appearing by Mallory and other histological methods, to be chromophobes, are found to belong to cyanophil series.

The method has been applied with good cytological results to the hypophysis of sheep and goats.

Solutions required:

A. Helly Fixative containing 5% of neutralized formalin.

B. Lugol's iodine.

C. Sodium thiosulphate 5% aqueous.

D. Hotchkiss' periodic Fuchsin.

E. Hotchkiss' reducing rinse (*Solution C as page* 184).

F. Feulgen's Fuchsin.

G. *Celestin blue* (*Lendrum and Mcfarlane*)

Celestin blue	0·25 gm.
Iron alum	2·5 gm.
Distilled water	50 ml.
Glycerine	7 ml.

Dissolve the alum in the water: then add the celestin blue, and boil for three minutes. Cool and filter; then add the glycerine.

H. Toluidine blue 0·5% aqueous.

I. Orange G .. .. 2 gm.
Phosphotungstic acid 5% aqueous.. 100 ml.
Allow to stand for 48 hours; filter before use.

Technique:

1. Human and larger animal glands are bisected in the horizontal plane with a sharp knife, and the two halves are fixed and embedded separately. Rat hypophyses are fixed *in situ* after removal of the brain and overlaying meninges, by immersing the whole base of the skull in a beaker of the fixative.

2. Fix in Helly for eighteen hours (man, goat, sheep), or two to four hours (rat).

3. Wash from six to twelve hours in running water.

4. Dehydrate, clear and embed in paraffin wax.

5. Cut sections 4·5μ (rat), or 5·5μ (man).

6. Fix sections to slides; remove paraffin wax with xylol and pass through the usual descending grades of alcohol to distilled water.

7. Immerse in solution B for three minutes.

8. Immerse in solution C (sodium thiosulphate) for three minutes by which time the natural colour of the sections should have been restored and mercurial precipitate removed from the fixative.

9. Wash well with water.

10. Rinse with 70% alcohol.

11. Immerse in periodic acid (solution D) for five minutes.

12. Wash with 70% alcohol.

13. Immerse in Hotchkiss' reducing rinse (solution E) for one minute.

14. Wash with 70% alcohol.

15. Immerse in Feulgen's Fuchsin for ten to thirty minutes.

16. Wash in running water for ten to thirty minutes.

17. Stain in the celestin blue solution one half to three minutes.

18. Rinse in water.

19. Stain in the toluidine blue (solution H) for one half to three minutes.

20. Wash in running water for five minutes.

21. Stain in the phosphomolybdic orange G (solution I) for ten seconds.

22. Wash in water for five to thirty seconds, until a yellow tinge is just visible to the naked eye.

23. Dehydrate through the usual graded alcohols.

24. Clear in xylol and mount.

Note: In place of Toluidine blue, Iron Haematoxylin may be used at step 19 *in which case it will be necessary to differentiate quickly before washing in running water* (*step* 20).

Results:

Beta granules in the cyanophils and a number of granules and vesicles in cells which stain as chromophobes by other methods, are magenta to deep red: the colloid is magenta. Alpha granules of acidophils, orange yellow, and erythrocytes are a shade more yellow.

Nuclei: are blue-black.

Reference: Pearse, Anthony G. Everson (1950), *Stain Tech.*, 25, 95–102.

PERIODIC ACID - FEULGEN FUCHSIN (Hotchkiss)

For staining polysaccharide structures in fixed animal or plant tissue preparations

Solutions required:

A.	Periodic acid	0·4 gm.
	Distilled water	45 ml.
	Sodium acetate M/5	5 ml.
B.	Distilled water	10 ml.
	Periodic acid	0·4 gm.

Sodium acetate M/5 .. .. 5 ml.
Absolute alcohol .. .. 35 ml.

Note: This solution, which deteriorates after a few days, should be kept in an amber bottle.

C. *Reducing rinse:*

Potassium iodide .. .. 1 gm.
Sodium thiosulphate .. .. 1 gm.
Distilled water .. .. .. 20 ml.

Dissolve; then add with stirring:

Absolute alcohol .. .. 30 ml.
Hydrochloric acid 2N .. .. 0·5 ml.

Note: A precipitate of sulphur is slowly formed and this may be allowed to settle out, or the solution may be used immediately.

The solution loses its acid reaction on keeping for some time, and it should be tested with litmus paper; if the reaction is no longer acid a few drops of N/2 hydrochloric acid should be added until an acid reaction is obtained.

D. Feulgen's fuchsin.

E. *Sulphite wash water:*

Distilled water .. .. .. 45·5 ml.
Hydrochloric acid, pure, conc. .. 0·5 ml.
Potassium metabisulphite .. 0·2 gm.

Technique:

Any fixative may be used but for glycogen or other easily soluble polysaccharides, alcohol is the best fixative as it does not dissolve such substances, and Solution B should be used in place of Solution A.

1. The sections or smears are brought down to alcohol; then immersed in Solution A for five minutes.

2. Pour off solution; flood with 70% alcohol.

3. Immerse for five minutes in Solution C.

4. Flood with 70% alcohol; pour off; then immerse in Solution D for fifteen to forty-five minutes.

5. Wash two or three times in Solution E.

6. Wash thoroughly in distilled water.
7. Dehydrate; clear and mount in the usual manner.

Results:

The following are stained (red) intensely by this reaction: muscle glycogen, liver, hyaluronic acid, gastric mucin, umbilical cord polysaccharide, chitin, crude serum albumin, crude casein, pneumococcus type III polysaccharide, Friedlander type B polysaccharide, algin, lemon pectin, gum arabic, gum tragacanth, glycerine, serine, dihydroxyacetone, ribose, arabinose, a-glycerophosphate, mannitol, tartaric acid, gluconic acid; while the following take up the stain with moderate intensity: starch, glucuronic acid, pneumococcus type I polysaccharide, pneumococcus type II polysaccharide; and the following are weakly stained: cellulose, crystalline serum albumin, crystalline egg albumen, glucose, glucosamine, glucose-l-phosphate, galactose, maltose, saccharose, xanthosine, adenosine, muscle adenylic acid and phlorizin. Tryptophane is coloured brown, and the following do not take up the stain: ribonucleic acid, desoxyribonucleic acid, cellobiose, inositol, malic acid and tyrocidine.

Notes:

(*a*) Plant tissues are brilliantly stained, in general revealing cellulose or cellulose-like walls of the individual cells and stored carbohydrate such as starch granules, particularly in the region of the chloroblasts if these are present. The cell walls of freshly cut potato reveal every fold, wrinkle or tear.

(*b*) Accumulations of polysaccharides are less common in animal preparations, but mucin, because of its polysaccharide content, is strongly stained.

(*c*) *If it is desired to demonstrate glycogen, Solution B should be used in place of Solution A.*

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PHLOXIN - HAEMATOXYLIN

For hyaline, in animal tissues

Solutions required:

A. Ehrlich haematoxylin solution.
B. Phloxin 0.5% in 25% alcohol.

C. Lithium carbonate 0·1% aqueous.

Technique:

1. Stain in Ehrlich haematoxylin for five minutes.

2. Wash in water; then stain one half to one minute in the phloxine solution.

3. Wash in tap water; then decolorize in the lithium carbonate solution.

4. Wash in tap water; then dehydrate in the usual manner and mount in balsam.

Results:

Fresh hyaline appears as red droplets and threads; while older hyaline is pink to colourless; nuclei, blue.

PHLOXIN - METHYLENE BLUE

Rapid smear technique for Negri bodies in brain tissue (J. R. Dawson's method)

Solutions required:

A. Phloxin 2% aqueous.

B. Methylene Blue (Loeffler).

Technique:

1. The brain to be examined should be removed as quickly as possible; then small segments, 3 to 4 mm. thick, are cut from Ammon's horn perpendicular to its long axis and placed in a Petri dish. Cut away adjacent tissue, leaving only the horn.

2. Place a segment, cut surface downwards, on the small end of a new one-inch cork; then with a matchstick, wipe peripheral tissue downward and outward, so that the segment is more firmly attached to the cork and the grey matter containing the pyramidal cells bulges upwards. Press this gently against a scrupulously clean slide, and make a smear by repeating this process along the whole length of the slide. This operation should be carried out rapidly before the tissue commences to dry.

3. Fix immediately by immersing in pure methyl alcohol for five to ten minutes.

4. Rinse in running water; then stain in the phloxin solution from two to five minutes.

5. Wash in running water; then stain in methylene blue (Loeffler) for ten to thirty seconds.

6. Decolorize in 80% alcohol; then dehydrate in 95% alcohol and two changes of absolute alcohol.

7. Clear in xylol and mount in balsam.

Note: The slides should be handled with forceps throughout to prevent the preparation being spoiled by coming into contact with the fingers.

Results:

Pyramidal cells, blue; Negri bodies, bright red to reddish brown.

PHLOXIN - METHYLENE BLUE - AZUR B

For normal and pathological animal tissues

This is a rapid modification of Mallory's original method reducing the staining time from one hour or more to one to two minutes, and permitting good staining with formalin fixed tissues which is not possible with Mallory's original method which was designed for Zenker-fixed material. Colophony differentiation is obviated, and Phloxin is not washed out as in the original Mallory technique.

Solutions required:

A. Phloxin .. .. 0·5 gm.
Acetic acid 0·2% aqueous .. .. 100 ml.
Filter before use.

B. Methylene blue .. .. .. 0·25 gm.
Azur B .. .. 0·25 gm.
Borax.. .. .. 0·25 gm.
Distilled water .. .. .. 100 ml.

C. Acetic acid 0·2% aqueous.

Technique:

1. Fix sections to slides: remove paraffin and pass through the usual descending grades of alcohol to distilled water.
2. Immerse in solution A for one to two minutes.
3. Rinse well in water.
4. Stain for one half to one minute in solution B.
5. Destain partially in solution C.
6. Differentiate in three washes of 95% alcohol.
7. Dehydrate with two changes of absolute alcohol.
8. Clear in two changes of xylol.
9. Mount in synthetic medium (Permount, D.P.X. or Clearmount, etc.)

Results:

Nuclei and bacteria are stained blue with some metachromasia. Collagen and other tissue elements are bright pink to red. Erythrocytes, bright scarlet.

Reference: Thomas, John T. (1953), *Stain Tech.*, **28**, no. 6. 311—312.

PHLOXIN - TARTRAZINE

(A. C. Lendrum's technique)

A general histological stain and for the demonstration of inclusion bodies

Note: This technique, in which use is made of a stable phloxine solution, and which affords the advantage of brilliant demonstration of certain inclusion bodies, is superior to Masson's erythrosin-saffron, which is less readily prepared and which deteriorates fairly rapidly.

Solutions required:

A. Haemalum (Mayer).

B. Calcium chloride 0·5% aqueous. 100 ml.
Phloxine .. .. 0·5 gm.

C. Tartrazine, saturated in cellosolve.

Technique:

1. Stain for five to ten minutes with the haemalum, examining under the microscope at intervals, until the desired depth of staining has been attained.

2. Wash and blue in tap water or in saturated aqueous lithium carbonate.

3. Stain in the phloxine solution for half an hour.

4. Rinse quickly in water.

5. Drain off excess water and replace with tartrazine solution (Solution C, as above), using a dropping bottle to control the differentiation.

Note: The tartrazine replaces the phloxine from collagen. As tartrazine is readily soluble in water, slight overstaining is recommended before dehydration.

6. Rinse in 60% alcohol followed by 95% alcohol.

7. Dehydrate with absolute alcohol.

8. Clear in xylol and mount.

Results:

Kurloff bodies in guinea pig's lung are well shown. Inclusion bodies of a number of virus-containing tissues show retention of phloxine.

Note: Fixatives containing mercuric chloride give the best results.

Abstract *J. Path. & Bact.*, 59, 399–404, 1947, Lendrum, A. C.

PICRO - NIGROSIN

For eleidin and keratin

Solutions required:

A. Picric acid, saturated, aqueous.

B. Nigrosin 1% aqueous.

C. Terpineol .. .. 1 volume
Origanum oil .. .. .. 1 volume

Technique:

1. Tissues should be fixed in 10% formalin, and frozen sections employed.
2. Sections are stained for five minutes in the picric acid solution.
3. Rinse in distilled water.
4. Stain in the nigrosin solution for one minute.
5. Wash in distilled water.
6. Rinse in 95% alcohol.
7. Clear in Solution C.
8. Mount in balsam or in Cristalite.

Results:

Eleidin, blue-black. Keratin, bright yellow.

PROTARGOL - GALLOCYANIN (Foley)

For nerve fibres, sheaths and cells

Solutions:

A. Protargol .. .. 1% aqueous.
(Prepared by sprinkling the protargol powder on the surface of the water and leaving it to dissolve.)

B. Protargol 1% aqueous .. .. 50 ml.
Alcohol 95% .. .. .. 50 ml.
Pyridine pure .. .. .. 0·5 ml.

Note: The quantity of pyridine may be varied between 0·1 ml. and 2 ml. The higher concentrations facilitate the staining of thin fibres, whereas cell bodies and dentrites are better demonstrated with the lower proportions of pyridine.

C. Boric acid .. .. 1·4 gm.
Sodium sulphite anhydrous .. 2 gm.
Hydroquinone .. .. .. 0·3 gm.
Acetone .. .. 15 ml.
Distilled water .. .. .. 85 ml.
Dissolve each reagent in the above order in the water adding the next only after the previous one has been dissolved entirely.

D. Brown gold chloride .. .. 0·2 gm.
Distilled water .. .. .. 100 ml.
Glacial acetic acid .. .. 1 ml.

Note: Solutions A, B, C and D must be stored in dark bottles.

E. Oxalic acid 2% aqueous.

F. Sodium thiosulphite 5% aqueous.

G. Gallocyanin solution (Einarson).

H. Phosphotungstic acid 5% aqueous.

I. Aniline Blue 1% aqueous .. 1 ml.
Fast Green FCF .. .. .. 0·5 gm.
Orange G .. .. .. 2 gm.
Glacial acetic acid .. .. 8 ml.
Distilled water .. .. .. 91 ml.

Note: This solution should be diluted 2 : 3 with distilled water before use.

Technique:

Note: Metallic instruments must not be used in the following procedures which should be carried out in a darkened room. Sections should be stained in black embryo dishes or in tubes covered all round with thick brown or black paper.

Fix in 10% formalin and embed in Celloidin or L.V.N. Sections are cut 15 to 25μ in thickness.

1. Immerse for twenty-four hours in ammoniated alcohol (conc. ammonia 1 ml., alcohol 50% 99 ml.).

2. Drain well and transfer to Solution A for six to eight hours at 37° C.

3. Drain well and transfer to Solution B in another staining dish, for twenty-four to forty-eight hours at 37° C.

4. Rinse for about five to ten seconds in 50% alcohol; then reduce with Solution C for about ten minutes.

5. Wash in several changes of distilled water; then tone in Solution D for ten minutes.

6. Wash in several changes of distilled water; then transfer for one to three minutes in Solution E, afterwards rinsing in distilled water.

7. Immerse for three to five minutes in Solution F; then wash thoroughly in distilled water.

8. Counterstain overnight in Solution G.

9. Wash thoroughly in distilled water then immerse in Solution H for thirty minutes.

10. Without washing transfer section to diluted Solution I for one hour; then wash with 70% alcohol and differentiate the counterstain in 95% alcohol.

11. Transfer to normal butyl alcohol; clear in cedarwood oil and mount.

Results:

Nerve fibres and neurofibrils, blue-black. Nissl bodies, pale blue. Nuclei, blue-black with silver and gold if a higher percentage of pyridine was used in solution. Myelin sheaths, bright yellow. Connective tissues, various shades of blue and green.

PURPURIN

For calcium deposits in pathological tissues

Solutions required:

A. Purpurin, saturated in absolute alcohol (about 0·7%).

B. Sodium chloride, reagent grade, 0·75% aqueous.

Technique:

1. Fix material in 90% alcohol.

2. Dehydrate; clear; embed in paraffin wax.

3. Fix sections to slides; de-wax and pass through the usual descending grades of alcohol to distilled water.

4. Stain in the purpurin solution for about ten minutes.

5. Immerse in sodium chloride solution for about five minutes.

6. Rinse with 70% alcohol until the stain ceases to come away in clouds.

7. Rinse with 90% alcohol and dehydrate with absolute alcohol.

8. Clear in xylol and mount in balsam.

Results:

Calcium deposits are stained red.

QUINCKE REACTION

For haemosiderin

Solutions required:

A. Ammonium sulphide solution, concentrated .. .. .. 1 volume
Absolute alcohol .. .. 3 volumes

B. Basic fuchsin 0·5% in 50% alcohol.

Technique:

1. Tissues are fixed in neutral formalin 10%, or in absolute alcohol, and embedded in paraffin wax or Celloidin in the usual manner.

2. Bring sections down to distilled water, then immerse them from two to forty-eight hours in the ammonium sulphide solution.

3. Rinse thoroughly in distilled water.

4. Counterstain in the basic fuchsin solution for five to twenty minutes.

5. Wash in water; drain well; rinse in 80% alcohol.

6. Differentiate and dehydrate in absolute alcohol; clear in xylol and mount in Canada balsam or in Cristalite.

Results:

Haemosiderin, dark brown to black.

RHODAMINE B - ANILINE METHYLENE BLUE

For splenic and lymphoid tissues

Solutions required:

A. Methylene Blue, 2% alcoholic .. 10 ml.
Aniline water .. .. .. 15 ml.
Distilled water .. .. .. 30 ml.

B. Rhodamine B 1% aqueous .. 2·5 ml.
Distilled water .. .. .. 47·5 ml.

C. Solution A (above) .. .. 3 volumes
Solution B (above) .. .. 7 volumes

Technique:

Tissues are fixed in Zenker-Formol and embedded in paraffin wax.

1. After removal of mercurial precipitate by treatment with iodine in the usual manner (*see* page 28) sections are stained two to three hours in Solution C; then washed rapidly with absolute alcohol.

2. Clear in xylol; mount.

Results:

Basophile protoplasm, blue. Chromatin, violet blue. Nucleoli, red. Connective tissue, faintly stained yellowish red. Muscle, yellowish red. Erythrocytes, bright red. Acidophile granules of leucocytes, bright red. Hyalin and granules of Russell, bright red. Nuclei of the small lymphocytes are faintly stained violet.

SAFFRON

For connective tissue

Note: Saffron is the dried stigmata of crocus sativus, and should not be confused with safranin, which is an aniline dye.

Solution required:

A. Saffron .. .. 2 gm.
Distilled water .. .. .. 100 ml.

Boil gently for an hour; allow to cool; then filter; add 1 ml. of 40% formaldehyde and 1 ml. of 5% tannic acid to the filtrate.

Note: Saffron solution deteriorates after a few weeks, and it is best to prepare the solution in small quantities, as required.

B. Delafield or Ehrlich haematoxylin.

C. Erythrosin, 1% aqueous.

Technique:

1. Fix small pieces of tissue in Bouin, Zenker-formaldehyde or in mercuric-formaldehyde.

2. Wash; dehydrate; embed.

3. Sections are stained for five to ten minutes with Delafield or Ehrlich haematoxylin; rinse in water.

4. Blue in tap water in the usual manner or in 1% sodium phosphate (Na_2HPO_4).

5. Stain for two to five minutes in 1% aqueous erythrosin.

6. Rinse quickly with water.

7. Differentiate with 70% alcohol for a few seconds, controlling under the microscope, until the collagen fibres are nearly colourless.

8. Rinse in water; stain for five minutes in saffron solution prepared as above; rinse with water.

9. Wash rapidly first with 70% alcohol then with absolute alcohol; clear in xylol and mount.

Results:

Nuclei are stained blue. Cytoplasm, varying shades of red. Muscle, pink. Elastic fibres, pink. Collagen, yellow.

Improved differentiation of most cells and tissues is obtained by employing this method in place of haematoxylin and eosin.

SAFRANIN - CRYSTAL VIOLET - FAST GREEN - ORANGE 2

(S. S. Kalter's " Quadruple Stain for Animal Tissues ".) Abstract from *J. Lab. & Clin. Med.*, 28, 995–7, 1943.

This technique, which is a development of Flemming's triple stain, is particularly useful for histology students

Solution required:

A. Safranin, O .. .. .. 0·2 gm.
Formalin (40% formaldehyde) .. 4 ml.
Sodium acetate .. .. .. 0·5 gm.
Alcohol 50% .. .. .. 100 ml.

B. Crystal violet 0·5% aqueous.

C. Fast green F.C.F. - Orange G saturated in clove oil

D. Orange 2 saturated in clove oil.

Technique:

1. Any fixative may be employed, but if Bouin is chosen, remove picric acid with a few drops of saturated lithium carbonate in the 70% alcohol of dehydration series. Should a fixative containing mercuric chloride be chosen, then it will, of course, be necessary to remove mercuric precipitate in the usual way.

2. After fixation, wash, dehydrate, clear and embed in paraffin wax.

3. Cut sections no thicker than 7·5μ and fix to slides, avoiding the use of glycerine albumin, which will cloud the stain.

4. De-wax with xylol; then pass through absolute, 90% and 70% alcohol to water.

5. Stain in the safranin solution for twenty-four hours.

6. Rinse with water.

7. Stain with the crystal violet for one to two minutes.

8. Wash with water.

9. Immerse in 50% alcohol for two minutes.

10. Immerse in 95% alcohol for two minutes.

11. Immerse in the fast green-orange 2 (Solution C) for five minutes.

12. Differentiate, examining under the microscope, until the connective tissue is stained to the desired depth of green.

13. Immerse in clove oil for ten minutes.

14. Transfer to Orange 2 in clove oil for ten minutes.

15. Differentiate, examining under the microscope at intervals, until the desired depth of staining has been achieved.

16. Immerse for ten minutes each in two changes of xylol.

17. Mount in balsam.

Results:

Nuclei, red. Nucleoli, purple or purplish red. Nuclear membrane, dark red. Cellular cytoplasm, pink to red, except in Henle's loop (light green). Connective tissue, green. Elastic fibres, yellow. Fibroblasts, green with purple nuclei. Muscle, reddish brown. Erythrocytes, orange. Polymorphonuclears show purple nuclei.

SAFRANIN - WATER BLUE (Unna)

For collagen fibres

Solutions required:

A. Safranin O 1% aqueous.

B. Water Blue 1% aqueous.. .. 10 ml.
Tannic acid 33% aqueous .. 10 ml.
This solution must be freshly prepared.

Technique:

1. Tissues should be fixed in 1% aqueous picric acid or in absolute alcohol, and Celloidin sections should be employed.

2. Stain for ten minutes in the safranin solution; then wash thoroughly in water.

3. Stain for ten to fifteen minutes in Solution B.

4. Wash thoroughly in distilled water.

5. Clear in Bergamot oil; then mount.

Results:

Collagen fibres are stained blue, while nuclei are red

SCARLET R - ETHYLENE GLYCOL

An improved technique for staining fat, etc. in animal tissues, its chief advantages being: (*a*) Excellent differentiation without loss of stain out of the lipid particles. (*b*) A stable solution which does not dissolve lipid materials. (*c*) Sections are not shrunken but remain pliable. (*d*) More intense staining of fat.

Solutions required:

A. Ethylene glycol, pure, anhydrous.

B. Scarlet R .. .. 1 gm.
Ethylene Glycol, pure, anhydrous.. 100 ml.
Heat the ethylene glycol to 100–110° C. on a hot plate or in an oven, or over the bunsen flame, taking care that it does not catch fire; then add the stain and stir until all or most of it is dissolved. Filter when cold.

C. Ethylene glycol, pure, anhydrous.. 85 ml.
Distilled water .. .. .. 15 ml.

D. Ehrlich or Delafield haematoxylin.

Technique:

1. Fix material in 10% formalin and cut frozen sections.

2. Wash sections in water for two minutes or longer to remove the formalin.

3. Dehydrate the sections by agitating gently in pure anhydrous ethylene glycol for three to five minutes.

4. Immerse the sections in the stain (solution B) for two to three minutes, with gentle agitation.

5. Differentiate by agitating gently in 85% ethylene glycol (solution C) from one to ten minutes, controlling by examination under the microscope while the preparation is still wet.

6. Transfer to distilled water for three to five minutes.

7. Counterstain with Ehrlich or Delafield haematoxylin.

8. Wash well in tap water.

9. Mount in glycerine jelly.

Results:

Nuclei, blue. Fat, orange to red. Cholesterol, red. Normal myelin, unstained. Fatty acids, unstained.

SCARLET R

For staining fat, etc. in animal tissues

Solutions required:

A. Scarlet Red, saturated in equal volumes of acetone and 70% alcohol.

B. Ehrlich or Delafield haematoxylin.

Technique:

1. Tissues are fixed in formalin and frozen sections are employed.

2. Sections are immersed for a second in 70% alcohol; then stained for two to five minutes, in the Scarlet R solution.

3. Wash quickly in 70% alcohol, and transfer to distilled water.

4. Counterstain with Ehrlich or Delafield haematoxylin.

5. Wash well in tap water; mount in glycerine or glycerine jelly.

Results:

Nuclei, blue. Fat, orange to red. Cholesterol, red. Normal myelin, unstained. Fatty acids, unstained.

SILVER CARBONATE - ORCEIN - ANILINE BLUE - FAST GREEN

For demonstrating reticulin, elastin and collagen in the same tissue sections

Solutions required:

A. Celloidin 0·5% in equal vols. of Ether and Absolute Alcohol.

B. Pot. Perman. 0·25% aqueous.

C. Oxalic Acid 5% aqueous.

D. *Silver carbonate, Hortega (Foot's modification)*
Silver nitrate 10% aqueous .. 10 ml.
Lithium carbonate, saturated aqueous .. .. 10 ml.
Shake well; then allow to stand for ten minutes or so in a 25 ml. measuring cylinder.

Pour off the supernatent fluid, then transfer the precipitate to a 100 ml. measuring cylinder, and add about 75 ml. distilled water, shake well; allow to settle; then pour off the fluid and add a second lot of distilled water. This process should be repeated three or four times.

Finally add 25 ml. of distilled water to the precipitate and add 28% ammonia solution drop by drop until the precipitate is almost dissolved.

Make up to 100 ml. with 90% alcohol.

Filter and warm to 50° C. for 15 minutes before using.

E. Neutral Formalin 40% .. .. 20 ml.
Distilled water .. .. .. 80 ml.
Buffer to pH 7·0.

F. Gold chloride 0·2% aqueous.

G. Sodium Thiosulphate 5%.

H. Orcein 1% in 70% alcohol .. 100 ml.
Hydrochloric acid, conc. .. .. 1 ml.

Picro Aniline Blue

I. Aniline blue aqueous .. .. 0·1 gm.
Picric acid, saturated aqueous .. 100 ml.

or:

J. Picric acid, saturated aqueous .. 100 ml.
Fast Green FCF .. .. .. 0·2 gm.

Technique:

1. Material should be fixed in 10% formalin and embedded in paraffin wax by the standard technique.

2. Sections 4 to 5μ in thickness are fixed to slides, dewaxed and immersed in xylol for five minutes.

3. Wash and immerse in absolute alcohol for two minutes.

4. Immerse in solution A for five minutes (Celloidin).

5. Drain slides for one minute.

6. Immerse in 80% alcohol for five minutes.

7. Rinse in water.

8. Immerse in solution B (Pot. perman.) for five minutes.

9. Rinse in water.

10. Immerse in solution C (oxalic acid) for five minutes.

11. Rinse in tap water.

12. Wash in distilled water.

13. Immerse in the silver carbonate solution in an oven at 50° C. for ten to fifteen minutes. (Soln. D).

14. Rinse in distilled water.

15. Immerse in solution E (20% neutral formalin) for five minutes.

16. Rinse in tap water.

17. Tone in solution F (Gold chloride) for five minutes.

18. Rinse in tap water.

19. Immerse in solution G for two minutes.

20. Rinse in tap water.

21. Stain in solution H (Orcein) for ten to fifteen minutes in an oven at 37° C. or for one hour at room temperature.

22. Rinse in 70% alcohol, followed by tap water.

23. Stain in solution I (Aniline blue) for twenty to forty seconds or solution J (Fast green) for ten to twenty seconds.

24. Wash with 95% alcohol for six to eight seconds.

25. Rinse briefly with absolute alcohol.

26. Rinse with a mixture consisting of equal volumes of xylol and absolute alcohol until clear.

27. Rinse with several changes of xylol, and mount.

Results:

With Picro aniline blue

Elastic fibres	reddish brown
Collagen	blue
Reticulum	black
Muscle	blue-green
Nuclei	tan to brown
Erythrocytes	yellowish tan
Cytoplasm	pale blue

With Picro fast green

Elastic fibres	orange to reddish brown
Collagen	blue-green
Reticulum	black
Muscle	green
Nuclei	tan to brown
Erythrocytes	yellow to orange
Cytoplasm	light green

Reference: Lewis, Ann L., and Jones, Russell S., (1951), *Stain Tech.*, **26**, 85–7.

SILVER NITRATE - GOLD CHLORIDE - PARACARMINE (Da Fano)

For Golgi apparatus

A. Cobalt nitrate 1% aqueous .. 100 ml.
Formalin .. .. 15 ml.

B. Silver nitrate 1·5% aqueous.

(*N.B.:* This should be stored in an amber or blue glass bottle.)

C. Cajal's Reducer.
Hydroquinone 2% aqueous .. 100 ml.
Neutral formalin .. .. .. 15 ml.
Sodium sulphite anhydrous .. 0·5 gm.

N.B.: This solution should be freshly prepared.

D. Gold chloride 0·2% aqueous.

E. Sodium thiosulphate 5% aqueous.

F. Paracarmine (Mayer).

Technique:

1. Pieces of tissue no thicker than 3 mm. are fixed from two to eighteen hours in the cobalt nitrate formalin solution, according to the size and nature of the material.

2. Wash the tissue quickly in a large volume of distilled water·

3. Immerse in the silver nitrate solution in the dark for thirty-six to forty-eight hours.

4. Wash quickly in a large volume of distilled water.

5. Trim the tissue to a thickness not exceeding 2 mm.

6. Immerse in Solution C (Cajal's Reducer) for two to twenty-four hours in the dark.

Note: For most soft tissues about four hours will suffice.

7. Wash in several changes of distilled water.

8. Dehydrate, clear and embed in paraffin wax in the usual way.

9. Cut sections up to 8μ in thickness.

10. Fix sections to slides; de-wax and pass through descending grades of alcohol down to distilled water.

11. Tone sections on slides by immersing in the gold chloride solution for five to ten minutes.

12. Wash quickly in distilled water.

13. Fix in 5% sodium thiosulphate (Solution E) for ten to fifteen minutes.

14. Wash thoroughly in distilled water.

15. Counterstain with paracarmine for about ten minutes.

16. Rinse with 90% alcohol, followed by absolute alcohol.

17. Clear in xylol and mount.

Results:

Golgi apparatus stained black while cells are pink or red.

SILVER NITRATE - HYDROQUINONE

For the detection of gold in fixed tissues of experimental animals

Notes:

(*a*) The following technique must be carried out in the dark-room.

(*b*) Fixatives containing metals must be avoided.

(*c*) The use of metal instruments in handling the sections must be avoided.

Solutions required:

A. Gum acacia 10% aqueous, filitered 100 ml.
Silver nitrate, A.R. grade .. 2 gm.

Note: This solution should be prepared immediately before use.

B. Gum acacia 10% aqueous, filtered 100 ml.
Hydroquinone .. .. .. 1 gm.

Note: This solution should be prepared the day before it is required for use.

C. Solution A .. .. .. 10 ml.
Solution B .. .. .. 10 ml.
Citric acid 5% .. .. .. 0·5 ml.

Note: This solution should be prepared only when required for immediate use.

D. Sodium thiosulphate 5% aqueous.

Technique:

1. Small pieces of tissue are fixed in 20% formalin, and frozen sections are employed.

2. Rinse thoroughly in distilled water.

3. Immerse sections in Solution C and leave therein for five to ten minutes.

4. Plunge sections directly into the sodium thiosulphate solution, without prior washing, and leave therein for five minutes.

5. Wash thoroughly in several changes of distilled water.

6. Mount in Aquamount or in Farrant's medium.

Results:

The presence of gold is indicated by a black deposit in the cells.

SUDAN BLACK

For lipids (especially those that are not well coloured by Sudan III or IV)

(J. R. Baker's technique)

Solutions required:

A. *Formaldehyde-saline.*
Formalin (Formaldehyde 40%) .. 10 ml.
Sodium chloride 10% aqueous .. 7 ml.
Distilled water .. .. .. 83 ml.

Note: Keep a few pieces of marble chips in the solution to maintain neutrality.

B. Formalin (Formaldehyde 40%) neutral.

Note : Keep a few pieces of marble chips in the bottle.

C. Potassium dichromate 2·5%
aqueous .. .. 88 ml.
Sodium chloride 10% aqueous .. 7 ml.

Note: Keep a few pieces of marble chips in the bottle.

D. *Dichromate-formaldehyde.*
Solution B .. .. 1 volume.
Solution C .. .. 19 volumes.

E. Potassium dichromate 5% aqueous

F. *Gelatine for embedding.*
Gelatine powder .. .. .. 25 gm.
Water .. .. 100 ml.
Sodium p-hydroxybenzoate .. 0·2 gm.

Sprinkle the gelatine on to the water and leave it to soak for an hour, afterwards warming in an incubator maintained at 37° C. until all the gelatine has dissolved, then strain through muslin while still warm.

Note: If Sodium p-hydroxybenzoate, which is added to prevent the growth of moulds and bacteria, is not available in the laboratory, then 0·25 to 0·5 gm. of Thymol should be used instead.

G. *Formalum* (*for hardening gelatine*)
Formalin (Formaldehyde 40%) .. 20 ml.
Potassium alum 5% aqueous .. 80 ml.
Keep marble chips in the bottle.

Note: Both gelatine blocks and gelatine sections may be preserved indefinitely in Formalum, which makes the gelatine very hard, thereby facilitating the cutting of thin sections which are non-sticky.

Important: Formalum must not be used in the acid haematein test for phospholipids (Baker 1946) as the alum would react with the haematein.

H. Sudan Black .. .. .. 0·5 gm.
Alcohol 70% .. .. .. 100 ml.
Boil for ten minutes under a reflux condenser; then cool and filter.

I. *Carmalum* (*Mayer*).

Technique:

1. Fix a piece of tissue not more than 3 mm. thick in the formaldehyde-saline for an hour.

2. Transfer, without washing, to the dichromate formaldehyde (Solution D) and leave for five hours.

3. Transfer, without washing, to 5% aqueous potassium dichromate and leave for about eighteen hours.

4. Leaving the tissue in the same solution, transfer to the paraffin oven at 60° C. for twenty-four hours.

5. Wash in running water for six hours.

6. Leave overnight in the melted gelatine in the oven at 37° C.

7. Cool the gelatine, preferably in a refrigerator.

8. Cut out a rectangular block containing the specimen.

9. Immerse the block overnight (or any conveniently longer time) in formalum, placing a marble chip in the capsule or tube.

10. Cut sections 8 to 10μ on the freezing microtome.

11. Transfer a section to 70% alcohol.

Note: It is best to transfer sections from fluid to fluid, up to stage 16 *in a Royal Worcester Porcelain thimble No. a.*4756, *size* 2.

12. Transfer to the Sudan black solution, and leave for $\frac{1}{2}$–4 minutes. (The best period is usually about $2\frac{1}{2}$ minutes.)

13. Wash in 70% alcohol for five seconds.

14. Wash in 50% alcohol for one minute.

15. Wash in water, sinking the section gently with a camel hair brush if it floats.

16. Transfer to Carmalum for two to three minutes. (The optimum time is usually three minutes.)

17. Rinse in distilled water.

18. Transfer the section to a fairly large dish, or a tongue jar of tap water, and leave for two minutes, or any conveniently longer time.

18. Wash again in another large bowl of water.

19. Transfer to a petri dish of water.

20. Float the section on to a slide.

21. Blot away excess water but do not allow the section to dry.

22. Mount in Farrant's medium, or in Aquamount.

23. Attach a clip to hold the coverslip to the slide: then leave overnight in the oven to harden the mounting media, before examining the preparation under the oil immersion objective.

Note: The slide may be examined after a quarter of an hour, if desired; then returned to the oven to complete the hardening.

Results:*

Lipids, dark blue or blue-black. Cytoplasm: colourless or pale grey-blue. Chromatin: pink or red.

Note: If the results are not good, another section should be tried with variations of the staining times.

Never attempt to judge the colouring until the section is mounted and examined under the oil immersion objective.

It is recommended that the technique be learned on the intestine of the mouse, as it is scarcely possible to fail with this. Cut out a piece of empty intestine about 1 cm. long and immerse in formaldehyde-saline for five minutes, then open it by a longitudinal cut from one end to the other, taking care not to do any unnecessary damage to the villi.

The section should be left only one minute in the Sudan black and two and a half minutes in the carmalum.

Reference: Baker, J. R. (1949), *Q.J.M.S.*, 90, 293–307.

* In the original paper the method refers to "Golgi bodies". Dr. J. R. Baker, to whom I am indebted for permission to include this abstract, informs me (March, 1955) that he regards the method as essentially for those lipids that are not well stained by other Sudan colours. In the original paper it is stated: "Simple Golgi bodies and externa: dark blue or blue-black. Golgi vacuoles: colourless". The changes are Dr. J. R. Baker's.

SUDAN BLACK

A specific stain for neutral fats

Solutions required:

A. Sudan black, saturated in 70% alcohol.

B. Carmalum (Mayer).

Technique:

Tissues are fixed at least three days in 10% formalin; then rinsed thoroughly in distilled water.

1. Frozen sections are immersed one minute in 50% alcohol; then one minute in 70% alcohol.
2. Stain for fifteen minutes to several hours in the Sudan black.
3. Rinse for a few minutes in 50% alcohol; then in distilled water.
4. Counterstain in carmalum for about three minutes; wash with distilled water; mount in glycerine jelly.

Results:

Neutral fat and myelin, blue-black to black; nuclei, red.

SUDAN BLACK - ETHYLENE GLYCOL

An improved technique for lipid staining, offering the advantage of a stable solution, excellent differentiation without loss of stain out of the lipid particles, and pliable unshrunken sections

Solutions required:

A. Ethylene glycol, pure, anhydrous.

B. Sudan black .. .. 1 gm.
Ethylene glycol, pure, anhydrous.. 100 ml.

Heat the ethylene glycol to 100–110° C. on a hot plate or in an oven, or over a bunsen flame, taking care that it does not catch fire; then add the stain and stir until all or most of it is dissolved. Filter when cold.

C. Ethylene glycol, pure, anhydrous.. 85 ml.
Distilled water .. .. .. 15 ml.

D. Carmalum (Mayer).

Technique:

1. Fix tissues for at least three days in 10% formalin.
2. Wash thoroughly in running water to remove the fixative.
3. Dehydrate the sections by agitating gently in pure anhydrous ethylene glycol for three to five minutes.
4. Immerse the sections in the Sudan black solution from fifteen minutes to one hour, agitating gently at intervals.
5. Differentiate by agitating gently at intervals with 85% ethylene glycol (solution C) from one to ten minutes, controlling under the microscope while the section is still wet.

6. Transfer the sections to distilled water for three to five minutes.

7. Counterstain with carmalum for about three minutes.

8. Wash with distilled water.

9. Mount in glycerine jelly.

Results:

Lipid substances or particles are stained blue-black to black. Nuclei, red.

Reference: Chiffelle, T. L., and Putt, F. A. (1951), *Stain Tech.*, **26**, no. 1, pages 51–6.

SUDAN BLUE

For demonstrating degenerated myelin

Solution required:

Sudan blue, saturated in 50% alcohol.

Technique:

1. Tissues should be fixed for at least three days in 10% neutral formalin.

2. Frozen sections are soaked one minute in 50% alcohol; then one minute in 70% alcohol; then stained from fifteen minutes to several hours in a saturated solution of Sudan blue in 50% alcohol.

3. Rinse for a few minutes in 50% alcohol; then in distilled water.

4. Counterstain in carmalum for three to five minutes.

5. Rinse in water; mount in glycerine jelly.

Result:

Degenerated myelin, blue.

SUDAN BROWN

For fat and for acute fatty degeneration not shown by Scarlet R

Solutions required:

A. Stock solution of Sudan brown saturated in isopropyl alcohol.

B. Mayer's acid haemalum.

Technique:

1. Fix tissues in 10% formalin.

2. Frozen sections are stained for five minutes in a diluted solution of Sudan brown prepared by mixing 6 volumes of the stock solution with 4 volumes distilled water.

(*Note:* This diluted solution keeps only for one day, and should, therefore, only be prepared immediately before use.)

3. Float out in tap water.

4. Stain for two to five minutes in Mayer's acid haemalum; then rinse in tap water.

5. Immerse in tap water or in 1% sodium phosphate (Na_2HPO_4) until the section turns blue.

6. Rinse in distilled water.

7. Mount in Apathy's gum syrup.

Results:

Fat, brown; nuclei, bluish grey; protoplasm, colourless.

SUDAN 2

For degenerating and intact myelin and fat

Solutions required:

A. Haematoxylin 1% in absolute alcohol, at least one to five days old .. .. 50 ml.
4% iron alum aqueous .. .. 50 ml.
This solution should be prepared immediately before use.

B. Borax .. .. 1 gm.
Potassium ferricyanide .. .. 5 gm.
Distilled water .. .. .. 100 ml.

C. Iron alum .. .. .. 0·5% aqueous

D. Sudan 2, saturated in isopropyl alcohol .. .. .. 30 ml.
Distilled water .. .. .. 20 ml.
Mix well and allow to stand for ten minutes before use.

Note: This solution deteriorates within three to four hours.

Technique:

1. Formalin-fixed frozen sections are stained forty minutes with Solution A at 56° C. in a covered dish in an oven.

2. Rinse in water and differentiate for one hour with Solution B.

3. Rinse in distilled water and immerse for ten minutes in Solution C.

4. Stain ten to twenty minutes in Solution D; then float out in water.

5. Mount in Apathy's gum syrup or in Aquamount.

Results:

Normal myelin, blue-black; nerve cells, grey; nuclei, deeper grey; red corpuscles, yellow to black; fats, orange yellow.

THIONIN (Ehrlich)

For mucin

Solution required:

Thionin (Ehrlich), saturated, aqueous .. .. .. 0·5 ml.
Distilled water .. .. .. 10 ml.

Technique:

1. Tissues should be fixed in Zenker's fluid, washed in running water, dehydrated, cleared, embedded and sectioned in the usual manner.

2. Sections are mounted on slides and the mercuric precipitate from the fixative is removed by the standard technique (*see* page 28).

3. Bring down to distilled water as usual; then stain from five to fifteen minutes in the thionin solution.

4. Dehydrate rapidly as otherwise the stain will be removed by the alcohol.

5. Clear in xylol and mount.

Results:

Mucin is metachromatically stained purple; while the basophil granules of the mast cells, Wharton's jelly of the umbilical cord, are purple; and the other tissue constituents are stained in varying shades of blue.

THIONIN (Ehrlich)

For the differential staining of entamoeba in sections

Solutions required:

A. Thionin 0·25% aqueous.

B. Oxalic acid 2% aqueous.

Technique:

1. Pieces of tissue are fixed in absolute alcohol and embedded in Celloidin in the usual manner.

2. Immerse in the thionin solution for three to seven minutes.

3. Differentiate in the oxalic acid solution for thirty to ninety seconds, controlling by examination under the microscope.

4. Rinse in water.

5. Rinse in 70% alcohol.

6. Dehydrate by rinsing in two changes of 95% alcohol.

7. Clear in terpineol and mount.

Results:

Nuclei of amoebae are stained a rich brown colour, while the nuclei of all other cells are stained blue.

THIONIN (Ehrlich)

For nerve cells and fibre tracts

Solution required:

Stain and fixative combined:

Thionin 0·5% in 10% formalin.

Technique:

1. Tissues are fixed and stained simultaneously by immersing in the above solution from a few days to three months.
2. Wash well in running water.
3. Dehydrate in ascending grades of alcohol in the usual manner.
4. Clear; embed in paraffin wax or in Celloidin.
5. Fix sections to slides and mount in Cristalite.

Results:

Cell bodies are stained blue, while the fibre tracts are red.

THIONIN (Ehrlich)

For demonstrating malignant cells in biopsy material

Solution required:

Thionin 1% aqueous.

Technique:

1. Stain frozen sections for ten to sixty seconds in the thionin solution.
2. Rinse in water.
3. Mount in tap water or in Aquamount.

Results:

Nuclei, blue to purple. Collagen, red. Elastic tissues, green.

TOLUIDINE BLUE

For mucus

Solution required:

Toluidine Blue 1·5% aqueous.

Technique:

1. Formalin-fixed material is embedded in paraffin wax in the usual manner.

2. Bring sections down to distilled water then stain for one or two minutes in the toluidine blue solution.

3. Wash with distilled water; drain well, then plunge the slide into two changes of 95% alcohol.

4. Dehydrate by immersing in two changes of acetone; then clear in xylol and mount.

Results:

Mucus, reddish violet; nuclei, blue; erythrocytes, yellow to greenish yellow.

TRICHROME STAIN

(G. Gomori)

A. Delafield or Ehrlich Haematoxylin.

B. Lithium carbonate 1% aqueous.

C. Alcohol 70% .. .. .. 97 ml.
HCl, concentrated .. .. .. 3 ml.

D. Picric acid 1% in 50% alcohol.

E. Phosphotungstic Acid 3%.

F. Light green, or Fast green FCF, or
Aniline blue .. .. 0·5 gm.
Neoponceau (Michrome) .. .. 1·5 gm.

G. Solution F .. .. 1 volume
Acetic acid 2% .. .. 3 to 4 volumes

Technique:

1. Fix tissues in Bouin or 10% Formalin.
2. Stain sections in the Haematoxylin solution for ten minutes.
3. Blue in the lithium carbonate solution.
4. Differentiate, if necessary, with the HCl alcohol for predominance of the green or blue shades, or in picric alcohol for predominance of red shades, in the final picture.
5. Immerse the preparation in 3% phosphotungstic acid for ten to fifteen minutes.
6. Wash gently under the tap for one minute.
7. Stain in solution G for five to twenty minutes.

Note: The time is not critical although results will be slightly different.

8. Rinse in 2% acetic acid.
9. Dehydrate, clear and mount.

Results:

Nuclei, blue. Cytoplasm, muscle fibres, red cells, etc., in shades of red. Connective tissue green or blue.

From personal communications with Professor G. Gomori of the Department of Medicine, University of Chicago, U.S.A., to whom I am indebted for permission to include this hitherto unpublished technique.

Note: Professor Gomori has used Woodstain scarlet, which is not available under that name in Britain. The British equivalent is Neoponceau and I feel that this synonym is more suitable in this case as the name Woodstain scarlet, indexed in literature on general biology, might suggest a botanical stain for woody tissues.

TRICHROME STAIN (Masson), Modified

For epithelium, pituitary and thyroid glands, nerve (normal and tumour), etc.

Solutions required:

A. Regaud's haematoxylin.

B. Picric acid saturated in 95% alcohol 20 ml.
Alcohol 95% .. .. .. 10 ml.

C. *Violamine-acid fuchsin:*

Violamine, R	0·7 gm.
Acid fuchsin	0·3 gm.
Glacial acetic acid	1 ml.
Distilled water	100 ml.

D. *Acetic fast green*

Fast green, F C F	3 gm.
Glacial acetic acid	2 ml.
Distilled water	100 ml.

Technique:

1. Tissues are fixed in Bouin, Regaud, Zenker or formalin, and embedded in paraffin wax.

2. Mordant sections on slides with 5% iron alum previously heated to 45° C.

3. Wash with tap water; stain for five minutes in Regaud's haematoxylin; then rinse with 95% alcohol.

4. Differentiate with picric alcohol; then wash with running tap water.

5. Stain for five minutes in Ponceau-acid fuchsin; then wash with distilled water.

6. Differentiate for five minutes in 1% phosphomolybdic acid; then without rinsing:

7. Flood the slide with solution D and leave for five to ten minutes.

8. Rinse with distilled water; then return to 1% phosphomolybdic acid for five minutes.

9. Leave in 1% acetic acid for five minutes.

10. Dehydrate in 95% alcohol, followed by absolute alcohol; clear in xylol and mount.

Results:

Nuclei, black. Argentaffin granules, black or red. Cytoplasm, vermilion. Collagen, green. Neuroglia fibrils, vermilion. Mucus, green. Keratin, vermilion. Intercellular fibrils, vermilion. Golgi apparatus, clear.

UREA SILVER NITRATE

For nerve fibres and nerve endings

Note: In this technique, nerve fibres and nerve endings of the peripheral and central nervous system are preferentially stained.

Applied to paraffin sections on slides, the technique gives rapid and constant results, and eliminates the necessity of gold toning.

The following fixatives are recommended:

(I).	Chloral hydrate	25 gm.
	Alcohol 50%	100 ml.
(II).	Formalin, undiluted (i.e. Formaldehyde 40%).	20 ml.
	Alcohol 95%	80 ml.
(III).	Ammonium bromide	2 gm.
	Formalin, undiluted (i.e. Formaldehyde 40%	15 ml.
(IV).	95% or Absolute Alcohol	
(V).	Bouin's fluid.	

Solutions, I, II, III and IV are satisfactory for Central Nervous System and nerve trunks.

Solution I has been used with satisfaction for striated muscle tissue.

Solution II is suitable for gland and smooth muscle tissue.

Solution V for gland and smooth muscle tissues and for embryos.

Tissues may also be fixed in 10% formalin with good results, but an excessive staining of connective tissue has been observed when this fixative has been employed.

Solutions required:

A.	Picric acid, saturated aqueous ..	50 ml.
	Mercuric cyanide	0·5 gm.
B.	Silver nitrate 1% aqueous	100 ml.
	Urea	25 gm.
	Solution A	3 drops
C.	Hydroquinone	2 gm.
	Urea	25 gm.
	Distilled water	100 ml.
	Sodium sulphite, anhydrous ..	10 gm.

Technique:

1. Fix the material in one of the above fixatives (I, II, III, IV or V) and embed in paraffin wax.

2. Fix sections to slides and remove paraffin wax with xylol.

3. Rinse with two changes of absolute alcohol.

4. Wash with 90% followed by 80% alcohol.

5. Immerse slides directly into solution B for one to one and a half hours at 50 to 60° C. in an oven.

6. Rinse quickly in two changes of distilled water.

7. Reduce by immersing in solution C for three minutes at 25 to 30° C., agitating the slides gently for the first two minutes.

8. Wash thoroughly in four or five changes of distilled water.

9. Wash with 50% followed by 70% and 80% alcohols.

10. Examine under the microscope while the preparation is still wet and if it is found that the staining is not complete, repeat step 5 using the original urea-silver nitrate solution and reducing the time to ten to fifteen minutes; then repeat steps 6, 7, 8 and 9.

11. Rinse with 95% alcohol.

12. Dehydrate with two changes of absolute alcohol.

13. Clear in xylol and mount.

Results:

Nerve fibres are stained from brown to black, while nerve endings are usually black, and nerve cells from yellow to brown. The background is usually yellow, but its appearance depends upon the kind of tissue and the fixative employed.

Reference: Ungewitter, L. H. (1951), *Stain Tech.*, **26**, p. 75.

VERHOEFF'S STAIN

For elastic fibres, nuclei and collagen

Solutions required:

A. Haematoxylin 5% in absolute alcohol .. .. 20 ml.

Ferric chloride (hydrated) 10% aqueous .. .. .. 8 ml.
Iodine solution (1 gm. iodine, 2 gm. KI, 50 ml. water) .. 8 ml.

Note: Solution A deteriorates after twenty-four hours.

B. Ferric chloride hydrated 2% aqueous.

C. Van Gieson stain.

Tissues should be fixed in Zenker or in 10% formalin: if the former is used mercurial precipitates are removed by the iodine in the staining solution and it is not, therefore, necessary to treat the sections or tissues with iodine before staining.

Technique:

Paraffin wax, Celloidin or L.V.N. may be used for embedding.

1. Sections are brought down to distilled water; then immersed in Solution A for one quarter to one hour until quite black.

2. Differentiate for a few minutes in Solution B, controlling by examination in water under the low-power objective.

3. Wash with tap water; then immerse in 95% alcohol to remove iodine.

4. Wash in tap water for five minutes; then counterstain in Van Gieson for three to five minutes.

5. Differentiate in 95% alcohol; then dehydrate.

6. Paraffin sections are cleared in xylol; Celloidin or L.V.N. in terpineol (after 95% alcohol).

7. Mount in Cristalite or in balsam.

Results:

Elastic fibres, intense blue-black to black. Nuclei, blue to black. Collagen, red. Other tissue elements, yellow.

WATER BLUE - ORCEIN - SAFRANIN

For demonstrating epithelial fibres

Solutions required:

A. Water blue .. .. .. 1 gm.
Orcein .. .. 0·75 gm.
Glycerine .. .. 20 ml.
Absolute alcohol .. .. 50 ml.
Acetic acid 5% .. .. .. 100 ml.

B. Eosin 1·25% alcoholic.

C. Hydroquinone 1% aqueous.

D. Safranin O, aqueous 1%.

E. Potassium dichromate 0·5%.

Technique:

1. Specimens of skin are fixed in 10% formalin and embedded either in paraffin wax or in Celloidin.

2. Bring sections down to distilled water; then stain for ten minutes in a mixture consisting of:

Solution A .. .. .. 10 ml.
Solution B .. .. .. 3 ml.
Solution C .. .. .. 3 ml.

3. Wash well in distilled water.

4. Stain for ten minutes in the safranin solution.

5. Wash thoroughly in distilled water.

6. Immerse in 0·5% potassium dichromate solution from ten to thirty minutes.

7. Wash in distilled water; dehydrate in absolute alcohol; then clear in oil of bergamot.

8. Examine under the microscope; then if necessary differentiate alternatively with absolute alcohol and oil of bergamot until the depth of the safranin stain has been reduced.

9. Mount in balsam or in Cristalite.

Results:

Epithelial fibres are stained red, while the nuclei are pale violet;

plasmasomes, red; cytoplasm, blue to violet; granules of the neutrophil leucocytes, sky blue; elastic fibres, red; collagen fibres, blue.

WEIGERT - FRENCH ELASTIN STAIN

(Moore's modification)

This modification, which is due to G. W. Moore, of the Central Histological Laboratory, Archway Hospital, London, gives greater selectivity than either Sheridan or Weigert elastin stains, and consistently excellent results are obtained provided the stain is properly prepared.

Solutions of Moore's elastin stain will keep for several years without deterioration.

The dry stain requires time and great care for its preparation; the majority of workers will, no doubt, wish to purchase the stain ready for use, but for those who have the time and prefer to prepare the stain themselves, the method is given below.

Preparation of the dry stain:

A. Ferric chloride, anhydrous, A.R. grade .. .. 30 gm.
Distilled water .. .. .. 65 ml.
Dissolve; then make up the volume to 100 ml. with distilled water.

Note: This solution must be freshly prepared.

B. Crystal violet .. .. .. 2·5 gm.
Basic fuchsin .. .. .. 2·5 gm.
Dextrin .. .. 1·0 gm.
Resorcin, pure .. .. .. 10·0 gm.
Distilled water .. .. .. 500 ml.

Note: The water must be heated to about 95° C. in a large evaporating basin, the dyes and dextrin added and stirred until dissolved. The resorcin is then added and the solution raised to boiling point with constant stirring.

Technique:

1. When Solution B begins to boil, add 62 ml. of Solution A slowly, in small portions at a time over a period of five minutes, with constant stirring.

2. Continue with the boiling and stirring for a further period of two or three minutes until a coarse precipitate is obtained.

3. After cooling filter by means of a Buchner funnel and flask attached to a suction pump.

4. Wash the precipitate with distilled water until the runnings are colourless and the filtrate a clear azure blue; this usually requires 8 to 10 litres distilled water.

5. The preparation is then dried overnight in an incubator, after removing the filter paper.

Preparation of the staining solution:

1. The dried elastin stain is now dissolved in 550 ml. absolute alcohol plus 1 ml. HCl in a 1-litre flask, the neck of which is plugged lightly with cotton-wool. Solution is effected by boiling gently for about thirty minutes on a water bath or electric hot-plate.

2. Cool; filter; add 19 ml. concentrated HCl; then shake well and allow to stand for at least twenty-four hours.

Staining technique:

1. Sections are brought down to distilled water; then treated with 0·5% aqueous potass. permanganate for five minutes.

2. Rinse and bleach with 5% aqueous oxalic acid; then wash in running water.

3. Transfer to elastin stain for at least two hours at 37° C. or for one half to one hour in an oven at 60° C.

4. Blot and treat with absolute alcohol for three to five minutes.

5. Rinse with water and counterstain for three minutes with neutral red (Jensen).

6. Rinse and differentiate the neutral red for a few seconds in absolute alcohol.

7. Rinse in distilled water, then pour on 0·5% picric acid aqueous and wash off immediately with running water.

8. Blot; dehydrate in absolute alcohol; clear in xylol and mount.

Results:

Elastic fibres, blue-black. Nuclei, red. Erythrocytes and muscle, yellow.

Notes: Best results are obtained after the stain has been kept in stock for several weeks, when it becomes perfectly selective and remains so indefinitely.

The use of Coplin's jars, which can be "topped-up" occasionally to make good loss by evaporation, is to be recommended.

The picric acid gives a beautiful contrast to the neutral red and enhances the appearance of the elastic fibres by causing them to stand out against a neutral background. Care must be taken when using it, however, as overstaining tends to give the red nuclei an unpleasant brownish tinge. It is perhaps advisable, until the technique has been mastered, to use neutral red only, ensuring that it is properly differentiated in absolute alcohol.

WEIGERT - PAL TECHNIQUE

For myelin sheaths in brain and spinal cord and for peripheral nerves and ganglia

Solutions required:

A. *Weigert's rapid fixative:*

Potass. dichromate	5 gm.
Fluorchrome powder	2 gm.
Distilled water	100 ml.

Dissolve by heat; cool and filter.

B. Haematoxylin 10% in absolute alcohol .. .. 10 ml.
Absolute alcohol .. .. 90 ml.

C. Saturated lithium carbonate, aqueous .. .. .. 7 ml.
Distilled water .. .. .. 93 ml.

D. Potass. permanganate .. .. 0·25%

E. Oxalic acid 1% .. .. .. 50 ml.
Potass. sulphite, anhydrous 1% .. 50 ml.

Technique:

1. Slices of the material 2 to 5 mm. thick are fixed in 10% formalin; then transferred to Solution A for four to seven days.

2. Wash in running water for several hours; then dehydrate and embed in Celloidin, L.V.N. or in paraffin wax, or cut frozen sections (in which case 2% ammon. bromide should be added to the formalin fixing solution).

3. Sections 10 to 20μ in thickness are stained twenty-four to forty-eight hours in a freshly prepared mixture consisting of 1 volume Solution B and 9 volumes Solution C.

4. Immerse for one half to three minutes in Solution D; then rinse in distilled water.

5. Differentiate in Solution E for one half to three minutes or until the white matter is blue-black, and the grey matter almost colourless.

6. Counterstain with safranin 1% aqueous, if desired, for one half to two hours according to thickness of sections.

7. Wash thoroughly in water.

8. Dehydrate; clear and mount.

Results:

Myelin sheaths, blue-black. Myelinated fibres, black or blue-black. Grey matter, white or slightly yellow. Other structures, unstained (unless a counterstain has been used).

WOOL GREEN - HAEMATOXYLIN - PONCEAU S

For connective tissue and muscle

Solutions required:

A. Picric acid, saturated in 70% alcohol.

B. Weigert's haematoxylin A.

C. Ponceau S 1% in 1% acetic acid aqueous.

D. Weigert's haematoxylin B.

E. Wool Green S 1% in 1% aqueous acetic acid.

F. Acetone and xylol, equal volumes of each.

Technique:

1. Immerse sections in the picric acid solution for two minutes.
2. Wash thoroughly in running tap water.
3. Stain for five to seven minutes in Weigert's haematoxylin A.
4. Rinse in water and stain for three to five minutes in the Ponceau S solution.
5. Wash in water.
6. Immerse in Weigert's haematoxylin B.
7. Wash thoroughly in water.
8. Stain for three to five minutes in the wool green solution.
9. Decolorize for two minutes with 1% acetic acid.
10. Pour off excess acid; rinse well in distilled water; drain and blot carefully.
11. Rinse well with acetone.
12. Rinse with two washings of Solution F.
13. Clear in xylol and mount.

Results:

Muscle and cytoplasm, red. Connective tissue and basement membranes, green to dark blue.

WRIGHT'S STAIN

For general differentiation of blood corpuscles; for malarial parasites; trypanosomes, etc.

This stain is extensively used in America instead of Leishman stain which is preferred by British workers.

Solutions required:

Formol-saline, neutral, buffered:

A.	Formalin (40% formaldehyde) ..	100 ml.
	Sodium chloride, A.R.	8·5 gm.
	Distilled water	1 litre
	Acid sodium phosphate, monohydrate	4 gm.
	Anhydrous disodium phosphate	6·5 gm.

B. Wright's stain.

C. Acetic acid, 0·08% aqueous.

Technique:

1. Fix pieces of tissue in Solution A for sixteen to forty-eight hours.

2. Dehydrate in the usual ascending grades of alcohol; clear and embed in paraffin wax.

3. Fix sections, not exceeding 5μ in thickness to slides; remove wax with xylol; pass through descending grades of alcohol down to neutral distilled water.

4. Stain for three to five minutes in a freshly prepared mixture consisting of one volume of Wright's stain and two volumes of neutral distilled water, in a stoppered staining jar.

5. Rinse with neutral distilled water.

6. Differentiate with the acetic acid solution, controlling by examination under the microscope, until the protoplasm of the cells is pink, and only nuclei are blue.

7. Wash with neutral distilled water.

8. Dehydrate quickly with absolute alcohol; clear in xylol; mount in Cristalite.

Results:

Erythrocytes, yellowish red. Polymorphonuclears, dark purple nuclei, reddish violet granules, pale pink cytoplasm. Eosinophiles, blue nuclei, red to orange-red granules, blue cytoplasm. Basophiles, purple to dark blue nuclei, dark purple to black granules. Lymphocytes, dark purple nuclei, sky blue cytoplasm. Platelets, violet to purple granules. Malarial parasites and Leishmania: chromatin, red; cytoplasm, blue. Trypanosomes: chromatin, red.

Note: The timing of the staining either before or after dilution may be altered to suit individual requirements.

Staining effects similar to Giemsa are obtained by staining for ten minutes in Wright's stain diluted with four times its volume of distilled water buffered to pH 6·5.

SECTION 3—BOTANICAL METHODS

(Normal and Infected Tissues)

(a) GENERAL TREATMENT OF TISSUES

Material must be killed and fixed immediately it is collected to ensure that tissues are preserved with as life-like an appearance as possible.

Small organisms such as unicellular algae, filamentous fungi, etc., may be placed directly into the killing fluid. Large objects must be cut at once into pieces not exceeding 5 mm. in any one direction, and care must be taken to avoid rough handling and pressure.

There are a great number of killing and fixing fluids to choose from and the reader should refer to the chapter on fixatives in this book as well as to standard text-books on botany such as Johansen or Chamberlain. The best general killing fluid, however, is 70% alcohol containing 0·1% glacial acetic acid; material should be immersed in this fluid from ten minutes to an hour.

After killing and fixing, material may be preserved, if it is not required for immediate examination, in 70% alcohol containing 20% glycerine, or in 10% formalin buffered to pH 7·0.

Delicate material should be stored in 85% alcohol containing 20 ml. glycerine per 100 ml. while tough hard material is best stored in 50% alcohol containing 20 ml. glycerine per 100 ml., but here again the reader is referred to standard text-books on botanical technique.

Material taken from the fixing and killing fluid or from the preserving fluid should be washed well in running water. Tissues may be dehydrated, cleared and embedded, as in the case of animal tissues. Alternatively, sections may be cut, without dehydration, clearing and embedding, either by means of a hand microtome, or by placing thin slices of the material in a slit cut in a piece of elder pith and cutting sections " freehand " with the razor edge facing away from the operator. In cutting freehand sections, the razor should be kept wet with 10% aqueous glycerine.

Glycerine (pure or diluted with water), or glycerine jelly should be employed for mounting temporary preparations after staining, while the preparation of permanent slides may be carried out in

the same manner as for sections of animal tissues, except that dehydration should be more gradual in order to avoid violent diffusion and consequent shrinkage and distortion of delicate tissues; the alcohols used should be graded 50%, 70%, 80%, 90%, 95% and absolute.

(b) MISCELLANEOUS MICROCHEMICAL TESTS

Note: In the following tests fresh material, in general, should be employed and thick sections, up to about 40 or 50μ are preferable to thin ones, particularly if the substances under test occur in small proportions.

Aldehydes

Solution required:

Diphenylamine 1% in concentrated sulphuric acid.

Technique:

1. Sections of strictly fresh tissue are placed directly into a drop of the reagent on a slide.
2. Heat gently until a green coloration appears.
3. Continue the heating for about five minutes.

Results:

If the green colour persists, formaldehyde is indicated but if the green turns to red, aldehydes, other than formaldehyde, are present in the tissue.

Aleurone

This occurs in the seeds of Poaceae

Solution required:

Eosin 2% in saturated alcoholic picric acid.

Technique:

1. Sections are immersed in a few drops of the reagent on a slide and examined under the microscope.

2. As soon as the ground substance of the aleurone grains appear blue, add a few drops of absolute alcohol, when the globules should be colourless and the crystals a yellowish green.

3. Remove excess alcohol; clear in clove oil and mount in balsam.

Results:

Protein (aleurone) crystals, yellow. Globules, pink. Ground substance, dark red.

Amygdalin

This occurs in the seeds of *Amygdalus; Pyrus crataegus* and related genera, as well as the leaves of *Prunus laurocerasus*

Solutions required:

A. Picric acid, saturated, aqueous.

B. Sodium carbonate 10% aqueous.

C. Potassium hydroxide 4% in 70% alcohol.

D. Ferrous sulphate 2·5% aqueous (prepared *without* heating).

E. Ferric chloride 20% aqueous.

F. Hydrochloric acid, conc. .. 1 volume
Distilled water .. .. .. 4 volumes

Technique (a):

1. Immerse sections in the picric acid solution for half an hour, on slides.

2. Wash with water; then add one drop of the sodium carbonate to a section.

Result:

A red coloration indicates the presence of amygdalin, and this is confirmed by the odour of hydrocyanic acid (almond odour), which is a decomposition product upon which tests for amygdalin are based.

Technique (*b*)*:*

1. Place sections in the sodium hydroxide (Solution C) for three to five minutes.

2. Mix equal volumes of the ferric sulphate and ferric chloride (Solutions D and E above) in a test-tube or small beaker and heat to boiling.

3. Immerse sections in the above mixture and leave them therein for five to ten minutes.

4. Place sections on a slide in a drop of the diluted hydrochloric acid (Solution F above).

Results:

A deep blue precipitate (Berlin blue reaction) indicates the presence of hydrocyanic acid which is indicative of amygdalin.

Amylodextrin

This product, which is intermediate between maltose and starch, is present in solution in storage organs where starch is hydrolysed. Its presence is indicated by the red coloration it produces with Gram's iodine solution

Anthocyanin

This occurs in the petals of the vast majority of blue and red flowers; such flowers owe their colour to anthocyanin

Solutions required:

A. Glacial acetic acid.

B. Ammonia solution.

Technique:

1. Place petals in a little neutral distilled water on slides.

2. Cover with thick coverslip (No. 2 or No. 3) and press out the coloured liquid.

3. Run in a drop of glacial acetic acid under the coverslip.

4. Take another slide and repeat the process but run in a drop of strong ammonia solution instead of acetic acid.

Results:

Anthocyanin is red in acid solution and blue violet to green in alkaline solution.

Arbutin

This occurs in Ericacae and Pyrolacae

Solutions required:

A. Nitric acid 10%

B. Ammonia solution.

C. Ferric chloride 5% aqueous.

Technique (*a*):

1. Place sections in the nitric acid solution.

2. Cover with a coverslip and examine under the microscope immediately.

Results:

Cells containing arbutin assume a dark orange colour, which rapidly changes to yellow which slowly disappears altogether.

Technique (*b*):

This depends upon the principle that arbutin is converted, on hydrolysis, to glucose and hydroquinone.

1. The dry sections are placed in a drop of water on a slide and heated gently for two or three minutes, when the arbutin sublimes in crystals.

2. Add a drop of ammonia solution when the arbutin crystals assume a rich brown colour.

3. Repeat the process adding a drop of ferric chloride solution instead of ammonia: a pale green colour confirms the presence of arbutin.

Asparagine

This occurs widely in the plant kingdom, but is most readily demonstrated in etiolated seedlings of Lupins and tubers of Dahlia

Solution required:

Cupric acetate 5% aqueous.

Technique:

1. Place strictly fresh sections in a drop of the cupric acetate on a slide and leave therein for ten to twenty minutes.

2. Add absolute alcohol slowly, a drop at a time, whilst examining under the microscope, until ultramarine spaerocrystals of copper asparagine become visible, indicating the presence of asparagine in the tissue.

Calcium

Solution required:

Oxalic acid 2% aqueous.

Technique:

1. Sections are placed directly onto a slide and flooded with the oxalic acid solution and left for half an hour exposed to the air.

2. Pour off some of the liquid; then add a coverslip.

3. Pipette a drop of absolute alcohol along one edge of the coverslip, so that the alcohol is drawn under the coverslip by capillary attraction. Examine under the microscope.

Results:

Calcium, if present, will be indicated by the small but easily visible crystals of calcium oxalate.

Calcium Oxalate

Solutions required:

A. Cupric acetate, saturated, aqueous.

B. Ferric sulphate .. .. .. 5 gm.
Acetic acid 20% aqueous .. 100 ml.

Technique:

1. Sections are placed directly into a drop of the cupric acetate solution on a slide and left therein for about ten minutes.

2. Examine under the microscope and if calcium oxalate crystals are present they will have dissolved and the oxalic acid diffused into the intracellular spaces where cupric oxalate is formed.

3. To test for the dissolved oxalate, add a few drops of the ferric sulphate (solution B) and examine under the microscope. The appearance of yellow ferrous sulphate crystals confirms the presence of calcium oxalate, in the tissue.

Callose

Solution required:

Lacmoid 1% alcoholic	0·1 ml.
Distilled water	25 ml.

Technique:

1. Immerse sections in the lacmoid solution for about fifteen minutes, when callose, if present, is stained a brilliant blue.

2. Mount in a drop of glycerine on a slide and examine under the microscope.

The following solubility tests may be employed to distinguish callose from other membrane substances:

(*a*) Soluble in copper oxide ammonia, while cellulose and hemicelluloses are insoluble.

(*b*) Swells but is insoluble in solutions of ammonia, sodium carbonate, potassium carbonate, whilst pectic acid is soluble in these reagents.

(*c*) Soluble in glycerine at 280° C. whilst cellulose and chitin are insoluble.

Carotin

Solution required:

Potassium hydroxide 20% in absolute alcohol.

Technique:

1. Place sections of fresh young green leaves in the potassium

hydroxide solution in a stoppered jar and leave in the dark until the chlorophyll is extracted.

2. Remove the sections from the jar and wash them for ten hours in running water.

3. Transfer to a large volume of distilled water and leave therein for an hour, afterwards transferring to a fresh lot of distilled water for a further period of one hour.

4. Transfer to slides; mount in glycerine.

Results:

Carotin, if present, appears as orange-red crystals. Xanthophyll will appear after two or three days as yellow crystals.

Cellulose

Solutions required:

A. Gram's iodine.

B. Sulphuric acid 75%.

Technique:

1. Place sections in a drop of Gram's iodine solution on a slide.

2. Cover with a coverslip; examine under the microscope, taking careful note of the location of the blue coloration.

3. Place a drop of the sulphuric acid solution along the edge of the coverslip and observe the swelling of the cellulose membranes as the sulphuric acid seeps under the coverslip, hydrolysing the cellulose to a colloid substance known as hydrocellulose.

Note: As certain other plant substances give a blue colour reaction with iodine it is important to note the locality of any blue colour which appears prior to the hydrolysation with the sulphuric acid.

Chitin

This is said to occur principally in the higher fungi.

Solution required:

A.	Potassium hydroxide	78 gm.
	Distilled water	68 ml.

Technique:

1. Heat the potassium hydroxide solution in an open beaker to boiling point.

2. Place the sections in the beaker which should now be covered with a clock glass, and continue the boiling for half an hour.

3. Remove the sections and wash in 90% alcohol.

4. Treat the section with Gram's iodine solution.

Results:

Chitin, if present in the tissue, will be indicated by a reddish-violet colour.

Chlorides

Occur in roots of *Daucus carota* and *Beta; Primula obconica; solanum*

Solutions required:

A. Silver nitrate 5% aqueous.

B. Nitric acid 1·5% aqueous.

Technique:

1. Sections, which must be cut with a scrupulously clean razor, are placed in a drop of the silver nitrate solution on a slide.

2. Examine, without a coverslip, under the microscope, when the silver chloride precipitate will appear black.

Note: The presence of chlorides in the tissue is indicated by a precipitate which is white to the naked eye.

3. By means of glass needles transfer the sections to a drop of the diluted nitric acid solution on another slide.

4. Examine under the microscope when it will be observed that the acid clears the sections sufficiently to allow localization of the reaction.

5. Repeat stage 3 (above); add a few drops of ammonia solution to the slide until the precipitate just dissolves.

6. Examine again after an hour when the precipitate will reappear in crystalline form.

Chlorophyll

Solutions required:

A. Potassium hydroxide 20% in pure methyl alcohol.

Technique:

1. Place sections directly onto a slide and add two or three drops of ether.

2. Add a few drops of the potassium hydroxide solution.

Results:

Chlorophyll immediately turns brown, afterwards changing back to green again.

Formic Acid

Solutions required:

A. Mercuric chloride 1% aqueous.

B. Hydrochloric acid, conc.

C. Potassium hydroxide 1% aqueous.

Technique:

1. Place fresh sections on slides; flood with the mercuric chloride solution and heat on a water bath for an hour.

2. Pour off excess mercuric chloride solution and wash with distilled water which has been acidified by the addition of 1 ml. hydrochloric acid conc. per 100 ml.

3. Place sections on slides; add one drop of 1% potassium hydroxide, and examine.

Results:

Where formic acid is present, the cells are blackened.

Glutathione

Solutions required:

A. Acetic acid 1%.

B. Ammonium sulphate, saturated, aqueous.

C. Sodium nitroprusside 5% aqueous.

Technique:

1. Place thin sections of strictly fresh tissue on slides.

2. Flood with 1% acetic acid and heat gently till vapour rises. This is most conveniently done by placing the slides over a corner of a tripod and applying the heat by means of a very small bunsen flame which should be held some distance away from the underside of the slide.

3. Transfer to a watch glass and rinse in the ammonium sulphate solution.

4. Immerse in a mixture consisting of 0·5 ml. of the sodium nitroprusside solution and 5 ml. of saturated ammonium sulphate, in a watch glass.

5. Agitate gently but thoroughly by rocking the watch glass for a few minutes.

6. Whilst watching the sections closely, add 1 ml. of ammonia solution.

Results:

If glutathione is present the cells will assume a red colour, which usually lasts only a second or so, with the addition of the ammonia solution.

Inulin

Occurs in bulbs of *Dahlia variabilis*, etc.

Solutions required:

A. Thymol 15% in absolute alcohol.

B. Chloral hydrate .. .. .. 10 gm.
Distilled water .. .. .. 4 ml.

Technique:

1. Fresh tissues are fixed in 70% alcohol for three or four days.

2. Place sections in alcohol on slides and add a drop of the chloral hydrate solution.

3. Examine under the microscope and observe concentric layers of inulin crystals, if present.

4. Add one drop of the thymol solution followed by concentrated sulphuric acid.

Results:

On addition of the last two reagents inulin crystals immediately become red, dissolving after a minute or so.

Iodine

Solutions required:

A. Starch 1% aqueous suspension.

B. Potassium nitrite 20% aqueous.

C. Hydrochloric acid conc. .. 1 ml.
Distilled water .. .. .. 19 ml.

Technique:

1. Place fresh sections in a watch glass containing 2 or 3 ml. of the starch suspension together with three or four drops each of Solutions B and C.

Results:

Iodine is indicated if the starch is coloured blue.

Iron

Note: The use of iron or steel instruments in the following technique should be avoided. The section razor, which must, however, be used, should be scrupulously clean.

Solutions required:

A. Hydrochloric acid concentrated.. 2 ml.
Distilled water .. .. .. 98 ml.
Potassium ferrocyanide 10% .. 2 ml.

B. Alum carmine.

Technique:

1. Sections of fresh material are taken from distilled water and immersed in Solution A (above) for one half to one hour.

2. Wash well with several changes of distilled water.

3. Stain the nuclei with alum carmine solution for a few minutes, then wash well with distilled water.

4. Dehydrate through ascending grades of alcohol as usual, clear in xylol; mount in D.P.X. or Cristalite.

Results:

Nuclei, red. Iron (if present), blue.

Lecithin

Solutions required:

A. Scarlet R (Botanical).

B. Delafield haematoxylin.

Technique:

1. Fat is removed from sections by immersion overnight in acetone, in a stoppered jar or well-corked tube.

2. Fix sections to slides; rinse in two changes of pure acetone.

3. Stain the scarlet R solution for about ten to fifteen minutes.

4. Wash quickly with 70% alcohol.

5. Wash in distilled water.

6. Counterstain with Delafield haematoxylin for two to ten minutes.

7. Blue and wash in tap water.

8. Mount in glycerine or Aquamount.

Results:

Lecithin (if present), red. Nuclei, blue.

Nitrates

Solution required:

Diphenylamine	1 gm.
Sulphuric acid, conc.	75 ml.
Distilled water	25 ml.

Add the sulphuric acid cautiously to the water in small portions (about 5 ml.) at intervals in a 250-ml. conical flask, swirling the contents round gently to ensure thorough mixing.

Do not on any account add the water to the acid as this will result in the acid flying back and causing serious injury to the face and hands, clothing, etc.

After the diluted acid has cooled somewhat, add the diphenylamine.

Technique:

1. Place sections on slides under coverslips.

2. Place two drops of the diphenylamine solution along one edge of the coverslip.

Results:

If nitrates are present a deep blue colour develops as the diphenylamine-sulphuric acid solution seeps under the coverslip and comes into contact with the section. After a few minutes, the section disintegrates and the colour changes to light brown.

Pectic Substances

Solution required:

A. Ruthenium red, 0·02% aqueous

Technique:

1. Stain with the ruthenium red for thirty minutes.

2. Mount in glycerine on a slide.

Results:

Pectic substances are stained red.

Phosphates

Solution required:

Ammonium molybdate 10% in concentrated nitric acid.

Technique:

Place sections on a slide in a drop of the reagent, and examine under the microscope.

Results:

Phosphates are indicated by small black-bordered yellow drops, which develop into spaerocrystals and afterwards into cubes and octahedrons.

Phytosterol

Solution required:

Gram's iodine.

Technique:

1. Place thick sections on slides and cover with concentrated sulphuric acid.

2. If phytosterol is present sections will assume a red coloration. Add a drop of Gram's iodine solution and mix by rocking the slide backwards and forwards gently.

Results:

The presence of phytosterol is confirmed when the colour changes from red, after the addition of the iodine, to violet; then blue and finally to yellowish red or brown.

Potassium

Solution required:

Sodium Cobalt Nitrite.

Sodium nitrite	7 gm.
Cobalt nitrate	4 gm.
Distilled water	13 ml.
Glacial acetic acid	2 ml.

Technique:

1. Sections of fresh tissue are placed in a drop of the above reagent on a slide and examined under the microscope.

Results:

The appearance of fine yellow crystals of potassium cobalt nitrite indicates the presence of potassium.

Proteins

Solutions required:

A. Potassium ferrocyanide .. .. 0·8 gm.
Acetic acid .. .. .. 100 ml.

B. Ferric chloride aqueous 5%.

Technique:

1. Immerse sections of fresh material in Solution A for an hour.
2. Rinse quickly with 60% alcohol.
3. Add a few drops of 5% ferric chloride.

Results:

A blue coloration indicates proteins.

Saponin

Method (a)

Place a drop of concentrated sulphuric acid on a section of fresh material.

If saponin is present in the tissue the section immediately assumes a yellow colour which changes to red after about half an hour, and later to violet or bluish green.

Method (b):

Solutions required:

A. Barium hydroxide, saturated aqueous.

B. Calcium chloride 5%.

C. Potassium dichromate 10% aqueous.

Technique:

1. To locate the sites of saponin immerse sections in the barium hydroxide solution for sixteen to twenty-four hours; then examine under the microscope and observe the insoluble colourless compound formed by the interaction of saponin and barium.

2. Wash well with the calcium chloride solution.

3. Cover with the potassium dichromate solution and watch the reaction under the microscope.

Results:

The insoluble substance first formed between the barium and saponin is broken down, the barium uniting with the chromium, forming barium chromate, which is identified by its yellow colour. Cells containing tannin assume a rich brown colour during the reaction.

Sodium

Solution required:

Uranium acetate, saturated aqueous.

Technique:

1. Sections are placed in a few drops of the reagent on a slide.

2. Add one drop of hydrochloric acid to the preparation.

3. Place uncovered slides in a desiccator to facilitate the slow evaporation of the reagents.

4. Examine at hourly intervals over a maximum period of eight hours.

Results:

Pale yellow rhomboidal or tetrahedral crystals (sodium uranium acetate) indicate the presence of sodium in the tissue.

Note: The presence of magnesium is indicated by large rhomboidal crystals, with this technique.

Sulphates

Solutions required:

A. Benzidine hydrochloride .. .. 1 gm.
Hydrochloric acid, conc. .. 3 ml.
Distilled water .. .. .. 97 ml.

B. Hydrochloric acid, conc. .. 10 ml.
Distilled water .. .. .. 90 ml.

C. Barium chloride 10%.

Technique (a):

Sections of fresh tissue are placed in a few drops of the benzidine solution on a slide and examined under the microscope.

Results:

Scales or small glistening needles (benzidine sulphate) indicate the presence of sulphates.

Technique (b):

For cereal seeds and other tissues which contain fat.

1. Immerse the material overnight in acetone in a stoppered jar or well-corked tube, to remove the fat.

2. Transfer to slides; wash in two changes of pure acetone; then allow the sections to dry on the slides.

3. Add one drop each of Solutions B and C (above) to the slide and examine under the microscope, under a high-power objective.

Results:

A granular precipitate (barium sulphate) indicates the presence of sulphates.

Tyrosine

Solutions required:

Sodium molybdate 1% in sulphuric acid, conc.

Technique:

1. Place sections on slide and cover with absolute alcohol.
2. Allow the alcohol to evaporate completely.

3. Cover the section with a few drops of the sodium molybdate and warm gently for a few minutes.

Results:

A deep blue colour turning to violet after a few minutes indicates the presence of tyrosine.

(c) STAINING TECHNIQUES

ACID FUCHSIN - AURANTIA

For differentiating between bacteria and mitochondria in sections of infected tissue

Solutions required:

A.	Chromic acid 1% aqueous ..	50 ml.
	Potass. dichromate 1% aqueous ..	50 ml.
	Neutral formalin	8 ml.
B.	Acid fuchsin	2 gm.
	Aniline water	10 ml.
C.	Aurantia	0·5 gm.
	Alcohol 70%	100 ml.
D.	Phosphomolybdic acid	1 gm.
	Sodium hydroxide 1% aqueous ..	10 ml.
	Distilled water	100 ml.

E. Polychrome methylene blue (Unna).

Technique:

1. Kill and fix for twenty-four hours in Solution A.

2. Wash for twenty-four hours in running water; then dehydrate and embed. Sections should be cut as thinly as possible.

3. After removing the paraffin wax from slides they are dipped into a very thin solution of Celloidin in equal parts of absolute alcohol and ether; then passed through absolute, 90%, 70% alcohol to distilled water.

4. Stain in Solution B, heated to about 80° C., for a few minutes, then wash in running water.

5. De-stain for a few seconds in Solution C; then wash in water.

6. Immerse in Solution D for a few minutes; then rinse in water.

7. Stain for a few minutes in Solution E, then wash with water.

8. Dehydrate very quickly with 95% and absolute alcohol; clear in xylol and mount.

Results:

Bacteria, deep violet blue. Mitochondria and plastids, red.

ACID RUBIN - AURANTIA - TOLUIDINE BLUE (Kull's Stain)

For starch grains and mitochondria in plant tissues

Solutions required:

A. Acid rubin 1% aqueous.

B. Aurantia 5% in 80% alcohol

C. Tannic acid 2% aqueous.

D. Toluidine blue 1% aqueous.

Technique:

1. Fix material in Regaud's fluid.

2. Stain for five minutes in solution A heated to about 60 or 70° C.

3. Differentiate with solution B, controlling by examination under the microscope.

4. Wash in water.

5. Immerse in solution C for 20 minutes.

6. Wash well in water.

7. Stain for five minutes in solution D.

8. Pour off excess stain and rinse with 70% alcohol.

9. Differentiate in 90% alcohol.

10. Dehydrate with absolute alcohol.

11. Clear in xylol, and mount.

Results:

Mitochondria are stained red, while starch grains are blue.

Note: This is an adaptation of one of Volkonsky's techniques.

Reference: Milovidov (1928), *Arch. Anat. Micros.*, **24**, 9.

ANILINE HYDROCHLORIDE

A simple and rapid method of demonstrating lignified tissues

Solution required:

Aniline hydrochloride 10% aqueous, freshly filtered before use.

Technique:

1. Place sections on slides and cover with a few drops of the aniline hydrochloride solution, and allow this reagent to act for about five minutes.

2. Pour off excess; place a drop of glycerine on the section and cover with a coverglass.

Results:

Lignified tissues are stained yellow while the other tissues remain unstained.

BASIC FUCHSIN, AMMONIACAL

For lignified walls and cutin

Solution required:

A. Basic fuchsin, 10% alcoholic.

B. Strong ammonia solution .. 25 ml.

Note: The fuchsin solution is added drop by drop to the ammonia until a yellowish colour is produced.

The fuchsin is decolorized by the ammonia and if too much fuchsin is added, then a few more drops of ammonia must be added so that the final product is pale yellowish in colour.

Technique:

1. Immerse sections in the solution in a stoppered jar for five to ten minutes.

2. Transfer sections to watch glasses containing absolute alcohol, for about five minutes, until the alcohol takes on a pink coloration.

3. Transfer sections to a fresh lot of alcohol and leave therein for about five to ten minutes.

4. Rinse and dehydrate in a fresh lot of absolute alcohol.

5. Clear in clove oil.

6. Mount in Canada balsam.

Results:

Lignified walls and cutin, intense red; remainder, colourless.

BORAX CARMINE (Grenacher)

For bulk staining prior to sectioning, and for small whole mounts

Solutions required:

A. Borax carmine, alcoholic (Grenacher).

B. Alcohol 70% .. .. .. 100 ml.
Hydrochloric acid, concentrated 0·25 ml.

Technique:

1. Immerse material in the carmine solution from one hour to four days, according to the bulk and nature of the material, until sufficiently stained.

2. Rinse with and immerse in acid alcohol (Solution B, above) until clear.

3. Wash with 70% alcohol.

4. Dehydrate with 95% alcohol, followed by absolute.

5. Clear in xylol.
6. Embed in paraffin wax.
7. Cut sections and mount on slides.
8. De-wax with xylol.
9. Mount in Canada balsam in xylol.

Note: For whole mounts, after clearing in xylol (Stage 5), mount in balsam.

Results:

Nuclei are stained deep red, while cytoplasm is pink.

CHLORAZOL AZURINE

A simple double stain, non-fading, and particularly suitable for elementary class work

Solutions required:

A. Formaldehyde 40% .. .. .. 5 ml.
Acetic acid 50% .. .. .. 14 ml.
Absolute alcohol .. .. .. 63 ml.
Distilled water .. .. .. 20 ml.

B. Magnesium sulphate, crystals, 6% aqueous.

C. Chlorazol azurine, saturated aqueous.

D. Equal volumes of Solutions B and C.

Heat the mixture to 80° C. and stir well for five or ten minutes, taking care that this temperature is not exceeded by more than a few degrees.

Technique:

1. Sections of the material, fresh from the field, are transferred to solution A. Alternatively material may be stored in this fixative for several days before sectioning.

2. Take sections down to water.

3. Immerse sections in solution D (the solution should be shaken well immediately before use) in a jar or tube overnight, or for at least eight hours.

4. Wash well with tap water.
5. Wash quickly in 70%, 90% and 95% or absolute alcohol.
6. Mount directly in Michrome mountant or in Euparal.

Result:

Non-lignified cell walls, blue, Lignified cells, violet to red. Bark cells, orange.

Herbarium Specimens

Solution required:

Solution D, as above.

Technique:

1. Soak or boil the specimens in water.
2. Cut sections and stain directly with solution D, which has been well shaken immediately before use.

Note: Prolonged treatment in the fixatives employed bleaches the specimens sufficiently for the purpose of staining with chlorazol azurine; otherwise bleaching is not recommended.

Reference: Armitage, F. D., *J. Roy. Micr. Soc.*, 535, 826, 1.

I am indebted to Mr. F. D. Armitage, F.R.M.S., of The Laboratory, Green End Road, Boxmoor, Herts, England, for information he has given me regarding his use of this stain.

CHLORAZOL BLACK

A non-fading, general-purpose stain, which may be used for whole mounts as well as for sections. The stain requires no mordanting nor differentiation

Nuclei and chromosomes are stained black, cytoplasm and secreted products grey, by this stain, which has also been found useful for infected plant tissues.

Solution required:

Chlorazol black, saturated in 70% alcohol.

Technique:

1. Fix tissues in Bouin or Flemming and embed in paraffin wax.

2. Stain in a freshly prepared, unfiltered, alcoholic solution of chlorazol black (as above) for five to ten minutes.

3. Drain off excess stain; dehydrate; clear in xylol and mount.

Results:

Vascular plant: Cell wall, jet black; cytoplasm, greyish green; nuclei, yellowish green; nucleoli, deep amber to dark green.

Fern leaf: Cell wall, intense black; epidermis walls, heavy black; cytoplasm, light amber; nuclei, green; nucleoli, dark green; plastids, grey; suberized walls of midrib, dark amber; veins, dark amber.

Notes:

(*a*) The stain may be incorporated with Lactophenol.

(*b*) Benzyl alcohol may be used as a solvent of the stain, in which case the results are somewhat different.

(*c*) If it is desired to differentiate the stain dilute " Milton " (a proprietary antiseptic) may be used.

CHLORAZOL PAPER BROWN, B

For plant tissues

Material may be stained overnight, but in many cases, e.g. where the delicate cell contents are not germane to investigation, the equivalent depth of staining will be produced by boiling for one to two minutes in the staining solution. By employing the boiling technique finished slides can be obtained in five minutes.

Solution required:

Chlorazol Paper Brown, B, saturated aqueous.

Technique:

1. Sections are stained overnight in a saturated aqueous solution (about 3%) of the stain, or by boiling in this staining solution for one to two minutes.

2. Differentiate in 1% nitric acid.
3. Dehydrate in acetone; mount.

Results:

Epidermis, yellow. Cortex, yellow. Pericycle (lignified tissues). blood red. Zylem, salmon to blood red. Primary phloem, orange. Cambium, pale yellow. Secondary phloem, crimson. Sieve plates, some very bright crimson, others orange. Pith, amber.

Abstract: (1947) *Stain Tech.*, Vol. 22, 4, 155–6, B. Vercourt.

COTTON BLUE - SAFRANIN

For fungal hyphae in woody tissues

Solutions required:

A. Cotton blue 0·5% in Lactophenol.
B. Lactophenol.
C. Safranin 1% aqueous.

Technique:

1. Stain thin sections with solution A, which has been warmed to about 35° C., for five to fifteen minutes.
2. Wash with Lactophenol to remove excess stain.
3. Wash with 70% alcohol to remove Lactophenol.
4. Counterstain with the safranin for about ten minutes.
5. Wash with 70% alcohol taking care that the safranin is not entirely removed.
6. Rinse rapidly in 90% alcohol.
7. Dehydrate with absolute alcohol.
8. Clear in xylol.
9. Mount in Canada balsam in xylol or in Cristalite.

Results:

Fungal hyphae, blue. Xylem, red.

Reference: Chesters (1934), *Ann. Bot.*, 48, 820.

CYANIN - ERYTHROSIN

A botanical stain for cellulose and lignified tissues

Solutions required:

A. Cyanin, 0·1% in 50% alcohol.
Note: This is a very costly stain.

B. Erythrosin 1% in 70% alcohol.

Technique:

1. Stain sections from five to twenty minutes in the cyanin solution, placing the slide under a petri dish lid, or an evaporating basin lined with a piece of damped filter paper to prevent evaporation of the stain.

2. Wash rapidly with 50% alcohol.

3. Stain from half to one minute in the erythrosin.

4. Rinse quickly in 50%.

5. Rinse quickly in 95% alcohol.

6. Dehydrate by rinsing quickly in two changes of absolute alcohol.

7. Clear in xylol, and mount.

Results:

Lignified tissues are stained blue, while cellulose tissues are red.

DELAFIELD HAEMATOXYLIN - CELLOSOLVE

For botanical tissues, particularly for cell walls

Solutions required:

A. Delafield haematoxylin.

B. Alcohol 70% .. .. .. 99·5 ml.
Ammonia solution (sp. gr. 0·880) 0·5 cc.

Technique:

1. Fix sections to slides and stain in Delafield haematoxylin for three to five minutes.

2. Rinse in tap water.
3. Decolorize for about thirty seconds in 70% alcohol.
4. Rinse in 70% alcohol.
5. Blue in Solution B (above).
6. Rinse well in distilled water.
7. Immerse in two changes of cellosolve for one minute in each.
8. Drain and mount in Canada balsam or in Cristalite.

Results:

Nuclei and chloroplasts are stained bluish purple; cytoplasm in a paler shade of purplish blue, or colourless, lignified and cutinized tissues are yellow. Unlignified tissues, bluish purple.

ERYTHROSIN - LACTOPHENOL

A general stain for botanical tissues

Solution required:

Erythrosin 1% in lactophenol.

Technique:

1. Place sections on slides and add a drop of the staining solution.
2. Cover with a coverslip and examine under the microscope.

Results:

Lignified and cutinized tissues are stained yellow while cellulose walls are unstained. Nuclei and dense protoplasmic contents are red. Cytoplasm of vacuolated cells appear pink, while chloroplasts, slime plugs in sieve, tubes, etc., are red.

GRAM'S IODINE

A general stain for botanical tissues

Solutions required:

A. Gram's iodine.
B. Glycerine 50% aqueous.

Technique:

1. Stain with Gram's iodine solution for a few minutes.

2. Pour off excess stain; rinse in distilled water.

3. Mount in the glycerine solution; cover with a coverslip and examine under the microscope.

Results:

Starch, navy blue. Proteins, brown. Cytoplasm, light brown. Nuclei, dark brown. Chloroplasts, brown or blue. Cellulose walls, faint yellow. Lignified walls, deep yellow.

HAEMATOXYLIN (Heidenhain) - ANILINE BLUE

For the differential staining of nuclei, cytoplasm and cell walls of angiosperm shoot apices

This technique which is due to Dr. J. G. Vaughan, Department of Biology, Chelsea Polytechnic, London, S.W.3, has been used with success on *Anagallis* and certain members of the Cruciferae. No mixing of the dyes has been observed in the apical meristem region, as occurs with other stain combinations, and the picture is clear and well defined, thereby facilitating study and photographing.

Solutions required:

A. Haematoxylin (Heidenhain), No. 1.

B. Haematoxylin (Heidenhain) No. 2.

C. Iron alum 2% aqueous.

D. Aniline blue alcohol, soluble (Michrome brand) .. .. 1 gm.
Methyl cellosolve .. .. .. 100 ml.
Dissolve by heating on a hot plate or a waterbath, taking care that the cellosolve does not catch fire. Allow to cool; then filter.

E. Methyl salicylate .. .. .. 25 ml.
Xylol .. .. 33 ml.
Absolute alcohol .. .. .. 42 ml.

F. Methyl salicylate .. .. .. 40 ml.
Xylol .. .. 20 ml.
Absolute alcohol .. .. .. 20 ml.

G.	Xylol	90 ml.
	Absolute alcohol	10 ml.

Technique:

1. Pass sections through descending grades of alcohol to distilled water.

2. Mordant in solution A for thirty minutes.

3. Rinse in distilled water.

4. Stain in solution B for twelve hours.

5. Rinse in distilled water.

6. Differentiate in 2% iron alum solution.

7. Wash in running water for one hour.

8. Dehydrate in 25%, 50%, 70%, 90% and two changes of absolute alcohol.

9. Stain in the aniline blue solution for ten minutes.

10. Remove excess aniline blue with absolute alcohol.

11. Rinse in solution E.

12. Clear in solution F for ten minutes.

13. Rinse in solution G.

14. Rinse in two changes of xylol and mount in Canada balsam in xylol, Clearmount, Cristalite or D.P.X.

Results:

Nuclei and cytoplasm are well stained by the haematoxylin, while cell walls are stained very satisfactorily by the aniline blue.

Note:

(*a*) The success or failure of the method is said to depend on the quality of the aniline blue.

(*b*) Ilford Special Rapid Panchromatic plates were used for the preparation of the photomicrographs which are reproduced in the original paper.

Reference: Vaughan, J. G. (1955), *Stain Tech.*, **30**, no. 2, 79–82.

HAEMATOXYLIN - BISMARK BROWN

For Phloem tissues of woody plants

Solutions required:

A. Iron alum 2% aqueous.

B. Haematoxylin 1% aqueous.

C. Bismark Brown 1% aqueous.

Technique:

1. Immerse sections in solution A for ten to twenty minutes.

2. Drain off excess solution; then wash in six or seven changes of distilled water.

3. Cover the preparation with solution B, placing the slide on the microscope stage.

4. Observe the progress of the stain under the low power objective and when the required depth of staining has been attained, pour off excess stain and rinse quickly in distilled water.

5. Rinse again with two or three changes of distilled water.

6. Immerse the preparation in solution C in a staining tube or jar for three to four hours, depending on the thickness of the sections.

7. Remove excess stain by washing well with water.

8. Wash with 50% alcohol, followed by 70% alcohol.

9. Wash with 90% alcohol.

10. Wash with two changes of absolute alcohol.

11. Clear in xylol, and mount in Cristalite or Clearmount or Canada balsam in xylol.

Results:

Stone cells of hard woods are stained cherry red; bast fibres are brilliant orange, whilst the ray cells and other parenchymatous tissues are a chestnut brown, and the middle lamellae dark blue. The bast fibres, parenchymatous tissues, and middle lamellae of Coniferophyta are stained as indicated, but the stone cells turn a vivid burnt orange.

HEIDENHAIN HAEMATOXYLIN - SAFRANIN

A general stain for plant tissue, algae, fungi, etc., to demonstrate histological and cytological structures

Solutions required:

A. Haematoxylin (Heidenhain) No. 1.

B. Haematoxylin (Heidenhain) No. 2.

C. Picric acid, saturated, aqueous.

D. Safranin 1% aqueous.

Technique:

1. Fix and dehydrate tissues and embed in paraffin wax.

Note: Algae and fungi should be treated by the Venetian turpentine method (*see* page 43).

2. Fix paraffin sections to slides; remove wax with xylol.

3. Pass through absolute alcohol and the usual descending grades of alcohol, down to distilled water.

4. Mordant in Heidenhain haematoxylin No. 1 (Solution A) from one half to two hours but no longer, unless the material is algae in which case as long as twelve hours may be necessary.

5. Wash for five minutes in running tap water.

6. Rinse well in distilled water.

7. Stain algae for at least twenty-four hours, or other material from five to seven hours, in Heidenhain haematoxylin No. 2 (Solution B).

8. Wash in running water for five minutes.

9. Differentiate in Solution C (picric acid) from twenty minutes to two hours, controlling by examination at intervals under the microscope.

10. Wash for half an hour in running tap water.

11. Rinse well in distilled water.

12. Counterstain in the safranin solution for five to ten minutes.

13. Rinse in distilled water.

14. Dehydrate as usual.

15. Immerse for five minutes in equal parts of absolute alcohol and xylol.

16. Transfer to and immerse in xylol for five minutes.

17. Mount in Canada balsam in xylol.

Results:

Chromosomes, black to purple. Centrosomes and pyrenoids, black to purple. Lignified, suberized and cutinized structures, unstained or only faintly stained. Archesporial cells and early stages of sporogenous tissue, grey.

IODINE GREEN - ACID FUCHSIN

A botanical stain for lignified tissues, and for chromosomes

Solutions required:

A. Iodine 1% aqueous.

B. Acid fuchsin 1% aqueous.

Technique:

1. Fix material in Nevashin's fluid.

2. Take sections through to distilled water in the usual manner.

3. Immerse for at least twelve hours in the Iodine green solution.

4. Wash and differentiate with distilled water, examining at intervals under the microscope while the preparation is still wet, until the non-lignified tissues retain only a faint green tint.

5. Counterstain for about five minutes in the acid fuchsin solution, controlling by examination under the microscope, while the preparation is still wet, taking care to ensure that the acid fuchsin solution is not allowed to act long enough to extract the Iodine green from the lignified tissues.

6. Rinse quickly in 90% alcohol.

7. Dehydrate quickly in two changes of absolute alcohol.

8. Clear in clove oil.

9. Wash with xylol.

10. Mount in Cristalite, Clearmount or in Canada balsam in xylol.

Results:

Chromosomes and nuclei, green. Plastin and cytoplasm, pink to red. Lignified tissues, green.

JOHANSEN'S QUADRUPLE STAIN

For plant tissue

Solutions required:

A.	Safranin O, 1% in cellosolve ..	50 ml.
	Alcohol 95%	25 ml.
	Distilled water	25 ml.
	Sodium acetate	1 gm.
	Formalin	2 ml.
B.	Crystal violet 1% aqueous.	
C.	Alcohol 95%	25 ml.
	Cellosolve	25 ml.
	Tertiary butyl alcohol	25 ml.
D.	Fast Green, FCF, saturated in equal parts of clove oil and cellosolve	20 ml.
	Alcohol 95%	60 ml.
	Tertiary butyl alcohol	60 ml.
	Glacial acetic acid	0·6 ml.
E.	Alcohol 95%	30 ml.
	Tertiary butyl alcohol	30 ml.
	Glacial acetic acid	0·15 ml.
F.	Orange G saturated in cellosolve	20 ml.
	Cellosolve	20 ml.
	Alcohol 95%	20 ml.
G.	Clove oil	10 ml.
	Absolute alcohol	10 ml.
	Xylol	10 ml.

Technique:

1. Paraffin sections are brought down to 70% alcohol; then stained for twenty-four to forty-eight hours in Solution A (overstaining is not possible).

2. Rinse in water; then stain ten to fifteen minutes in Solution B.

3. Rinse in water; then rinse for fifteen seconds in Solution C.

4. Stain ten to twenty-five minutes, according to the material and fixative, in Solution D.

5. Rinse briefly in Solution E.

6. Immerse for about three minutes in Solution F.

7. Rinse in Solution G.

8. Rinse in two changes of xylol; then mount in balsam.

Results:

Dividing chromatin, red; resting chromatin, purplish; nucleoli, red (occasionally violet); nucleoplasm, colourless or greenish; lignified walls, bright red; cutinized cell walls, reddish purple; suberized walls, red; cellulose cell walls, greenish orange; cytoplasm, bright orange; middle lamellae, green; starch grains, purple with green or orange halos (the colour of the halos soon becomes replaced by purple in some types of materials); plastids, purplish to greenish; invading fungal mycelium, green; the callose portion of the guard cells of the stromata bright red and the remainder purple; and Casparian strips, red; the remainder of the cell wall of the endodermis, yellow.

In sections of roots for the origin of the lateral roots, the cytoplasm of the latter should be stained green, with purplish nuclei, while the cytoplasm elsewhere should be orange with red nuclei.

The combination is exceptionally good for sections of lichens, as the algae are well differentiated, and also for *Puccinia graminis telia* and *Uredinia*.

From *Plant Microtechnique*, by D. A. Johansen, by courtesy of McGraw-Hill Book Company, Inc., New York.

JOHANSEN'S QUINTUPLE STAIN

For plant tissue composed of a variety of cell types, such as leaves, roots, stems and ovaries

Solutions required:

A. Safranin O, 1% in cellosolve .. 50 ml.
Alcohol 95% .. .. .. 25 ml.
Sodium acetate 4% .. .. 25 ml.
Formalin .. .. 1 ml.

B. Crystal violet 1% aqueous.

C. Absolute alcohol .. .. 25 ml.
Cellosolve .. .. .. 25 ml.
Tertiary butyl alcohol .. .. 25 ml.

D. Fast Green, FCF.. .. .. 0·5 gm.
Malachite Green .. .. .. 0·5 gm.
Cellosolve .. .. .. 100 ml.
Dissolve; then add:
Tertiary butyl alcohol .. .. 25 ml.
Absolute alcohol .. .. 25 ml.
Glacial acetic acid .. .. 1·5 ml.

E. Orange 2 .. .. 0·5 gm.
Cellosolve .. .. .. 58 ml.
Dissolve by warming gently, then add:
Clove oil .. .. 14 ml.
Absolute alcohol .. .. 14 ml.
Tertiary butyl alcohol .. .. 14 ml.

F. Terpineol, extra pure .. } equal volumes
Absolute alcohol ..
Tertiary butyl alcohol ..
Cellosolve .. ..
Methyl salicylate ..
Beechwood creosote ..

G.	Terpineol, extra pure ..	equal volumes
	Absolute alcohol ..	
	Tertiary butyl alcohol ..	
	Cellosolve	
	Methyl salicylate ..	
	Beechwood creosote ..	
	Toluol	
H.	Toluol	45 ml.
	Absolute alcohol	5 ml.

Technique:

1. Paraffin sections are fixed to slides, and taken down through the usual stages to 70% alcohol.

2. Stain for four to forty-eight hours in the safranin (Solution A).

Note: The staining time depends upon the material. Angiosperm and Pteridophyte stems, leaves and roots, usually require at least twenty-four hours. Weakly lignified or overchromated tissues may need forty-eight hours. Gymnosperm materials need anything from four to forty-eight hours. Ovaries and embryos require four hours.

3. Rinse thoroughly in water.

4. Stain for five minutes in the crystal violet (Solution B).

Note: This stain should be omitted on ovules, embryos and all parasitic and saprophytic fungi; in such cases proceed from stage 3 directly to stage 7.

5. Rinse in running water for a few seconds.

6. Rinse for a minute in Solution C.

7. Stain for five minutes in the fast green - malachite green (Solution D).

8. Rinse briefly in running water.

Note: The preparations will appear to be excessively overstained, but this does not matter.

9. Rinse in Solution C to which 1% acetic acid has been added.

10. Stain for five minutes in the orange 2 (Solution E).

11. Rinse in Solution F for a minute.

12. Rinse in Solution G.

13. Immerse in Solution H for five to ten minutes.

14. Pass through two changes of alcohol; then mount.

Notes:

(*a*) Isopropyl alcohol may be used, if desired, for every purpose for which ethyl alcohol is commonly used.

(*b*) The schedule at first glance appears to be complicated, but the actual application is quite simple.

(*c*) Slides should be agitated in all rinses and washes.

(*d*) The reason for mixing so many reagents together in Solutions F and G is that each effects certain stains and not the others.

(*e*) The fast green - malachite green (Solution D) is somewhat strong at first but gradually weakens as successive slides are passed through it; consequently, the time in this stage may be reduced to four minutes and that of the orange 2 (Solution E) in Stage 10 lengthened to ten minutes, then equalized, and later, the changes in time reversed as the orange becomes stronger after its initial weakness.

(*f*) Approximately three hundred slides may be passed through each 100 ml. of each staining solution (except the safranin (Solution A), which merely needs replenishment when required), and rinsed before replacement becomes necessary owing to contamination.

From personal communications with Professor D. A. Johansen, Pomona, California, U.S.A., to whom this technique and my thanks are due.

LACMOID - TANNIC ACID - FERRIC CHLORIDE

For phloem and contiguous tissues: the technique gives relatively stable preparations of critically stained materials

Solution required:

A. Tannic acid 1% aqueous.

B. Ferric chloride, hydrated 2% aqueous.

C. Sodium bicarbonate .. .. 2·5 gm.
Distilled water .. .. .. 50 ml.

Dissolve by shaking or stirring: do not apply heat.

D.	Solution C	20 ml.
	Distilled water	55 ml.
	Absolute alcohol	25 ml.
E.	Lacmoid	0·25 gm.
	Absolute alcohol	30 ml.
	Distilled water	70 ml.
	Solution C	3 to 5 ml.
F.	Solution C	20 ml.
	Distilled water	30 ml.
	Absolute alcohol	50 ml.
G.	Absolute alcohol Clove oil Xylene	equal volumes of each

Technique:

1. Fix material in formalin-acetic-alcohol.

2. Unembedded sections, 5 to 40μ in thickness may be employed.

3. Sections are taken from distilled water and immersed in 1% tannic acid for five to ten minutes.

4. Transfer to 2% ferric chloride for about five mins.

5. Wash in distilled water (three changes).

6. Examine under the microscope while the preparation is still wet: the colour of the walls should be medium to dark grey, and it may be necessary to repeat the above staining process to attain this result as some phloem tissues stain less readily than others.

7. Wash in three changes of distilled water.

8. Immerse in solution D for thirty minutes.

9. Transfer directly to solution E and leave therein for twelve to eighteen hours, or longer if desired as it is impossible to overstain.

10. Transfer to solution F from ten seconds to ten minutes depending upon the time at which the lacmoid destains.

11. Wash in 80% alcohol.

12. Immerse in 90% alcohol for two to three minutes.

13. Immerse in two changes of absolute alcohol for a total time of two to three minutes.

14. Wash in solution G for two to three minutes.

15. Immerse in two changes of xylol for a total time of two to three minutes.

16. Mount in D.P.X. or Clearmount or Cristalite mountant.

Results:

Callose is stained sky blue to greenish blue; lignified cellulose (in the xylem cells and in most cortical or phloem fibres and sclereids) blue. Cellulose walls, nuclei, slime and cytoplasm, light brown to greyish brown.

Reference: Vernon, J. C. and Gifford, E. M. (1953), *Stain Tech.*, **28**, 49–53.

LACMOID - MARTIUS YELLOW

For callose in pollen tubes

Solution required:

Lacmoid 0·01% .. .. .. 10 ml.
Martius Yellow 0·01% .. .. 10 ml.

Add a few drops of diluted ammonia (0·1 ml. conc. ammonia with 10 ml. distilled water) until the solution assumes an olive tint.

Technique:

1. Slender styles or ovaries are crushed between two slides while still wet. Larger styles or ovaries should be cut by hand into longitudinal sections, then crushed.

2. Stain two to five minutes in the Lacmoid - Martius yellow solution.

3. Mount in the stain and examine under the microscope, using a strong light.

Results:

Pollen tubes, blue. Background, light yellowish green.

LIGNIN PINK

A non-fading stain which is specific for lignin

The stain gives constant results with reasonably thin sections and is particularly suitable for routine or elementary classwork.

Overstaining with Lignin Pink is impossible, and it will not wash out with alcohol.

In combination with chlorazol black it offers a simple double staining technique as follows:

Solutions required:

Lignin Pink	0·5 gm.
Chlorazol black	0·5 gm.
Distilled water	100 ml.

Technique:

1. Reasonably thin sections are fixed to slides and immersed in the staining solution for twenty to twenty-five minutes.
2. Rinse in distilled water.
3. Rinse in 70% alcohol.
4. Rinse in 90% alcohol.
5. Dehydrate with absolute alcohol.
6. Clear in xylol and mount.

Results:

Lignin stands out, stained bright carmine colour, against the surrounding tissues which are stained black.

Reference: Cannon, H. G. (1941) *J. R. Mic. Soc.*, series III, **61**, parts 3 and 4.

MAGDALA RED - FAST GREEN

A differential stain for parasite and host tissues in botanical material

Solutions required:

A. Magdala red 2% in 80% alcohol (original)

B. Fast Green FCF-Clove Oil.

Technique:

1. Embed the material in paraffin wax in the usual manner.
2. Remove paraffin wax from the sections with xylol as usual.
3. Wash well with absolute alcohol.
4. Wash with 90% alcohol.
5. Stain for five to ten minutes with solution A.
6. Wash with absolute alcohol to remove excess stain.
7. Counterstain for two to five minutes in solution B.
8. Wash in absolute alcohol.
9. Clear in xylol, and mount.

Results:

Host tissues are stained green; parasite, red.

METHYL GREEN - PHLOXIN - GLYCERINE JELLY

A rapid method for the simultaneous mounting and double staining of pollen grains, differentiating functional from abortive grains

This technique can be employed for the evaluation of orchid seeds which contain naked embryo surrounded by integuments.

Solutions required:

A. Methyl Green, saturated in 50% alcohol.

B. Phloxin, saturated in 50% alcohol.

C. Glycerine jelly .. .. .. 50 ml.
Solution A .. .. $2\frac{1}{2}$ ml.
Solution B .. .. 2 ml.

Melt glycerine jelly on a water bath; then measure off in a pre-heated measuring cylinder the 50 ml. required and pour this amount into a pre-heated bottle to which, solutions A and B are then added and shaken in.

Note: The jelly should now be a port wine colour: it may be necessary to vary the proportions by adding more of one of the dyes to produce this colour.

Technique:

1. Place a small amount of pollen on the centre of a slide.

2. Wash by dropping on 70% alcohol to remove any adhering oils and resins.

Note: The alcohol will spread out and evaporate leaving a ring of sludge, which should be removed with a piece of tissue paper moistened with alcohol.

3. Repeat this process until no more sludge comes out.

4. Wash finally with 70% alcohol, and just before the last drop of alcohol evaporates completely, place 2 drops of hot stained glycerine jelly on the pollen and stir gently with a needle to ensure even distribution.

5. Cover with a coverslip, keeping the jelly under the coverslip hot by heating gently with a bunsen flame.

Note: Excessive heating will rupture many of the pollen grains.

6. Remove the flame; take off the coverslip and replace it with a clean, flamed coverslip.

7. Allow the slide to cool; then store in a cool place.

Results:

"Functional" pollen grains are fully expanded; exine and intine are stained green; cytoplasm red. Aborted grains are either shrunken and stained green, or expanded in varying degrees depending on the amount of non-autolysed cytoplasm present at the time of mounting, and stained a mottled, reticulate red.

Notes: The intensity of the Phloxin stain increases on standing, whereas the action of the methyl green is practically instantaneous. Preparations retain their brilliancy for about a year.

Reference: Owczarzak, Alfred (1952), *Stain Tech.*, **27**, no. 5, p. 249.

PHLOROGLUCINOL

An extremely sensitive test for lignin, particularly suitable for hydrophytes

Solution required:

Phloroglucinol 1% in 70% alcohol.

Technique:

1. Sections are placed on slides and flooded with the phloroglucinol solution which is allowed to act for about five minutes.

2. Pour off excess phloroglucinol and cover sections with a few drops of concentrated hydrochloric acid, cover with a coverslip and examine under the microscope.

Result:

Lignified tissues are stained red.

POLYVINYL LACTOPHENOL

For embedding brittle specimens of wood for sectioning. It is claimed that this technique has given successful results with wood dating back to the Roman era

In addition to softening the wood for cutting, this technique also clears the specimen and allows cell walls, which may have been blackened through carbonization, to become clear and translucent, thereby facilitating identification under the microscope.

Solution required:

Polyvinyl Lactophenol.

Technique:

1. Immerse blocks of wood cubed in the usual manner, in polyvinyl alcohol, and warm gently for thirty minutes.

2. Drain, and allow to cool for twenty-four hours.

3. Cut sections from blocks with a very sharp razor.

Results:

The wood has lost its brittleness and has acquired a soft pliable rubber-like nature.

Reference: Levy, J. F. L. (1953), *Nature*, **171**, 984.

SAFRANIN - ANILINE BLUE

For plant tissues; particularly suitable for gymnosperm ovules, archegonia, embryos and angiosperm stems and roots

Solutions required:

A. Safranin O, 2% in Cellosolve .. 100 ml.
Absolute alcohol .. .. .. 50 ml.
Sodium acetate 4% aqueous .. 50 ml.
Formaldehyde 40% .. .. .. 8 ml.

B. Picric acid 0·5% in 95% alcohol.

C. Ammonia solution (sp. gr. 0.88) .. 0·25 ml.
Absolute alcohol .. .. .. 100 ml.

D. Aniline Blue, alcohol soluble, saturated in equal volumes of cellosolve and absolute alcohol.

Technique:

1. Take sections through to 70% alcohol in the usual manner.

2. Stain from two to forty-eight hours, according to the nature of the material, in solution A.

Note: Gymnosperms require the minimum staining time.

3. Wash thoroughly with running water to remove the excess stain.

4. Dehydrate and differentiate carefully with solution B.

5. Immerse the preparation in solution C for half to one minute.

6. Dehydrate morphological material in 95% alcohol, or cytological preparations in absolute alcohol.

7. Counterstain for about one minute in a mixture consisting of equal parts of solution D and clove oil.

8. Clear in methyl salicylate and mount in Cristalite, Canada balsam or Emexel mountant.

Results:

Lignified and cutinized cell walls, nuclei and chromosomes, bright red. Cellulose cell walls and cytoplasm, blue.

Note: At certain developmental and formative stages, some cell walls are stained sharply with the safranin, while other portions will appear faint blue.

SAFRANIN - ANILINE BLUE

For plant tissues, particularly for chromosomes and cell walls

Solutions required:

A. Safranin 1% aqueous.

B. Aniline blue 1% in absolute alcohol.

Technique:

1. Paraffin sections are fixed to slides; de-waxed with xylol, and passed through descending grades of alcohol down to distilled water as usual; alternatively freehand sections may be employed.

2. Stain for fifteen minutes in the safranin solution.

3. Wash in distilled water.

4. Rinse in 70% alcohol, followed by 90%.

5. Rinse in absolute alcohol.

6. Stain for two minutes in the aniline blue solution.

7. Rinse quickly but thoroughly with two changes of absolute alcohol.

8. Wash with two changes of xylol.

9. Mount in balsam.

Results:

Chromosomes, red. Nucleoli, red. Cytoplasm almost colourless. Cellulose walls, blue.

SAFRANIN - DIANIL BLUE G

For the differential staining of Peronosporaceae

Solutions required:

A.	Phenol crystals	10 gm.
	Lactic acid	10 ml.
	Glycerin	20 ml.
	Absolute alcohol	20 ml.

B. Cotton blue 4B .. .. .. 0·02 gm.
Safranin .. .. 0·10 gm.
Solution A .. .. 100 ml.

C. Safranin 0·25% in clove oil,.

Technique:

1. Embed material in paraffin wax by the standard technique.
2. Fix sections to slides and remove paraffin wax with xylol as usual.
3. Wash well with absolute, followed by 90% alcohol.
4. Immerse in solution A for ten to fifteen minutes.
5. Transfer to solution B and leave therein for two hours.
6. Differentiate in solution A.
7. Wash with absolute alcohol.
8. Immerse in solution C for twenty to thirty minutes.
9. Differentiate in clove oil.
10. Clear in xylol, and mount in Canada balsam in xylol or in Cristalite, or in Emexel mountant.

Results:

Host tissue is stained red; Myelium blue.

Reference: (1928), *Lepik. Phtopath.*, 18, 869.

SAFRANIN - FAST GREEN IN CELLOSOLVE

A rapid, non-fading stain for botanical tissues in place of Safranin-light green-clove oil

Solution required:

2% Safranin - Fast Green FCF in cellosolve.

Technique:

1. Stain sections for five to ten minutes.
2. Rinse in cellosolve.
4. Mount in Canada balsam or in Cristalite.

Results:

Lignified tissues are stained red, while unlignified tissues and cytoplasm are blue-green. Nuclei, red. Chloroplasts, pink to red.

SAFRANIN - ACID FUCHSIN

For spermatozoids, zoospores, motile gametes, etc.

Solutions required:

A. Osmic acid 1% aqueous.

B. Safranin 1% aqueous.

C. Acid Fuchsin 1% aqueous.

Technique:

1. Place a drop of an aqueous suspension of the organism on a slide.

2. Fix by holding the slide for about one minute over osmic acid solution.

3. Allow the preparation to dry.

4. Stain for ten minutes to one hour in solution B.

5. Wash in water.

6. Remove excess water by draining and blotting the edges of the slide with filter paper.

7. Wash in 95% alcohol until only the nuclei retain the stain.

8. Stain in the acid fuchsin solution for ten to twenty seconds.

9. Wash rapidly in 70% followed by 90% alcohol.

10. Wash rapidly with two changes of absolute alcohol.

11. Clear in clove oil, followed by xylol.

12. Mount in Canada balsam in xylol, Cristalite, Clearmount or Emexel mountant.

Results:

Nuclei bright red. Cytoplasm bluish pink.

Reference: (1918), *Steil. Bot. Gaz.*, LVX, 592.

SAFRANIN - FAST GREEN, FCF

A non-fading stain, satisfactory for nearly every type of plant material except the algae

Solutions required:

A. Safranin O, alcoholic .. .. 1 gm.
Cellosolve .. .. .. 50 ml.
Alcohol 95% .. .. .. 25 ml.
Distilled water .. .. .. 25 ml.
Sodium acetate .. .. .. 1 gm.
Formalin .. .. 2 ml.

B. *Fast Green FCF:*

Fast Green FCF saturated in equal parts Cellosolve and absolute alcohol added in sufficient quantity to give a stain of the desired intensity when mixed with a mixture consisting of one part absolute alcohol and three parts clove oil.

Technique:

1. Paraffin sections are brought down to 70% alcohol, or free-hand sections up to 35% alcohol.

2. Stain in Solution A for two to forty-eight hours (gymnosperm material needs the minimum period); then wash off excess stain in running water for a minute or so.

3. Differentiate and dehydrate simultaneously with 0·5% picric acid in 95% alcohol.

4. Immerse in 95% alcohol to which four or five drops of ammonia have been added from a few seconds to two minutes, but no longer.

5. Dehydrate quickly with absolute alcohol.

6. Counterstain with Solution B for ten to fifteen seconds.

7. Wash off excess stain with clove oil then clear in a mixture consisting of two parts of clove oil, one part absolute alcohol, and one part xylol.

8. Remove the clearing mixture by washing for a few seconds each in three changes of xylol; then mount in balsam.

Results:

Nuclei, chromosomes, lignified and cutinized cell walls, brilliant red. Cytoplasm and cellulose cell wall, brilliant green. In some cell walls at certain developmental or formative stages, portions will be stained less sharply by safranin, and other portions weakly by the fast green.

SAFRANIN - FAST GREEN, FCF IN CELLOSOLVE

A rapid non-fading stain for botanical tissues in place of Safranin - light green - clove oil

Solution required:

Safranin - light green in Cellosolve.

Technique:

1. Stain sections in the safranin - light green - cellosolve for five to ten minutes.
2. Rinse in Cellosolve.
3. Mount in Canada balsam or in Cristalite.

Results:

Lignified tissues are stained red, while unlignified tissues and cytoplasm are blue-green. Nuclei, red. Chloroplasts, pink to red.

SAFRANIN - LIGHT GREEN - CLOVE OIL

A general stain for botanical tissues

Solutions required:

A. Safranin 1% in 50% alcohol.

B. Light Green - clove oil.

Technique:

1. Stain section in the safranin solution for ten minutes.
2. Rinse in two changes of 50% alcohol for thirty seconds in each.

3. Rinse in two changes of 70% alcohol for thirty seconds in each.

4. Repeat with two changes of 90% alcohol for the same time.

5. Immerse in two changes of absolute alcohol for two or three minutes in each.

6. Stain in light green - clove oil for one minute.

7. Rinse and wash in clove oil for about five minutes.

8. Examine under the microscope and if it is found that the safranin has been extracted by the alcohols, take the section down through the alcohols and restain with safranin.

9. Mount in balsam or in Cristalite mountant.

Results:

Cellulose tissues and cytoplasm are stained green. Lignified tissues and nuclei, red. Chloroplasts, pink.

SAFRANIN - PICRO ANILINE BLUE

A rapid and simple method of demonstrating hyphae in wood sections

Solutions required:

A. Safranin 1% in distilled water.

B. Aniline blue, water soluble .. 2·5 gm.
Distilled water .. .. .. 25 ml.
Picric acid, saturated, aqueous .. 100 ml.

Technique:

1. Stain one to three minutes in Solution A; then wash in water.

2. Flood sections with Solution B and heat over a flame until it begins to simmer; then pour off excess stain; allow the slide to cool before washing with water.

3. Wash with 70%, followed by absolute, alcohol; clear in clove oil; mount in balsam.

Results:

Lignified walls, red. Mycelia, clear blue. Areas where the wood is badly decayed may appear bluish, but the hyphae are always well defined.

SAFRANIN - TANNIC ACID - FAST GREEN

For roots and stems

Solutions required:

A. Tannic acid 1% in 50% alcohol.

B. Ferric chloride 3% in 50% alcohol.

C. Safranin 1% in 50% alcohol.

D. Fast green FCF in clove oil to which has been added 7% of absolute alcohol.

Technique:

1. Sections are fixed to slides, treated with 70% alcohol; then immersed in the tannic acid solution.

2. Immerse for about twenty seconds each in two lots of 50% alcohol.

3. Treat with the ferric chloride for about thirty to sixty seconds.

4. Wash with 50% alcohol.

5. Stain with the safranin solution for twenty-four hours, in a stoppered staining jar.

6. Wash with 50% alcohol.

7. Differentiate for about ten seconds in 70% alcohol.

8. Pass through 80%, 90% and absolute alcohol, allowing ten to thirty seconds in each.

9. Stain for two or three minutes in the fast green.

10. Pass through absolute alcohol, followed by xylol; then mount in Cristalite.

Results:

Nuclei, red. Cytoplasm, blue-green. Lignified walls, red. Cambial cell walls, black. Collenchyma walls, very dark red. Parenchyma cell walls, black.

SCARLET R or SUDAN 3 - ETHYLENE GLYCOL

An improved technique for staining fat in plant tissues, offering the following advantages: (*a*) The use of ethyl alcohol is obviated and sections remain pliable and unshrunken; (*b*) excellent differentiation without loss of stain from the fat; (*c*) More intense staining of fat.

Solutions required:

A. Scarlet R or Sudan 3 .. .. 1 gm.
Ethylene glycol, pure, anhydrous.. 100 ml.
Heat the ethylene glycol to 100–110° C. on a hot plate, or in an oven for preference, but if these are not available the bunsen flame will serve the purpose so long as care is taken to ensure that the ethylene glycol does not take fire. Stir in the dye until all or most of it is dissolved; then cool and filter.

B. Ethylene glycol .. .. .. 85 ml.
Distilled water .. .. .. 15 ml.

C. Delafield or Ehrlich haematoxylin.

Technique:

1. Fix tissues in 10% formalin.

2. Wash out fixative with water.

3. Dehydrate the sections by agitating gently in the pure ethylene glycol, anhydrous, for three to five minutes.

4. Immerse the sections in the staining solution (Scarlet R or Sudan 3) and agitate for two to five minutes.

5. Differentiate by agitating gently, at intervals, from one to five minutes in 85% ethylene glycol (solution B) controlling by examination under the microscope while the specimen is still wet.

6. Transfer the sections to distilled water and leave therein for three to five minutes.

7. Counterstain with Delafield or Ehrlich haematoxylin.

8. Wash well in tap water.

9. Mount in glycerine jelly or glycerine.

Results:

With Scarlet R: Nuclei, blue. Fat, orange to red. Cholesterol, red. Fatty acids, unstained.

With Sudan 3: Nuclei, blue. Fat, yellow to orange. Cholesterol, orange. Fatty acids, unstained.

SCARLET R

For staining fat in plant tissues

Solutions required:

A. Scarlet R, saturated in 70% alcohol.

B. Delafield or Ehrlich haematoxylin.

Technique:

1. Fix tissues in 10% formalin.

2. Sections are immersed for a second in 70% alcohol; then stained for two to five minutes in the Scarlet R.

3. Wash quickly and transfer to distilled water.

4. Counterstain with Ehrlich or Delafield haematoxylin.

5. Wash well in tap water; mount in glycerine or glycerine jelly.

Results:

Fat, orange to red. Cholesterol, red. Nuclei, greyish blue. Fatty acids, unstained.

SCHULZE SOLUTION (Chlor Zinc Iodine)

For cell walls, proteins and starch

1. Place sections on slides and pour on a few drops of Schulze solution.

2. Cover with a coverslip and examine under the microscope.

Results:

Starch, blue. Proteins, brown. Cellulose walls, violet. Lignified walls, yellow. Cutinized and suberized walls, yellow to brown.

TETRAZOLIUM SALT

For testing the viability of seeds

Solution required:

A. Tetrazolium salt 1% aqueous.

Technique:

1. Select the seeds at random and steep in tap water for 18 hours.

2. Section longitudinally through the embryo and place one half of each in a 7 cm. petri dish.

3. Pour the tetrazolium salt solution over the seeds and soak in the dark for 4 hours at 20° C.

4. Wash with tap water.

5. Examine seeds for staining.

Results:

Viable seeds are stained: others unstained.

Note: The method is not well adapted to certain seeds.

Reference: Cottrell, Helen J. (1948), *Ann. Appl. Biol.*, **35**, 123–31.

THIONIN - ORANGE G

For infected plant tissues

Solutions required:

A. Carbol thionin.

B. Orange G 0·2% in absolute alcohol.

Technique:

1. Fix paraffin sections to slides; pass through xylol and descending grades of alcohol down to distilled water in the usual manner.

2. Stain for an hour in the thionin solution. Alternatively, free-hand sections may be employed, in which case, the sections should be stained in the thionin for only five minutes.

3. Pour off excess stain, and rinse with 70% alcohol followed by 90%.

4. Rinse quickly in two changes of absolute alcohol.

5. Differentiate for one half to one minute with the orange G solution.

6. Pass through 90%, followed by 70%, alcohol.

7. Rinse well with water.

8. Rinse with 70% alcohol, followed by 90%.

9. Rinse quickly in two changes of absolute alcohol.

10. Pass through two changes of xylol.

11. Mount in balsam or in Cristalite.

Results:

Parasites are conspicuously stained violet-purple, with deep purple nuclei. Cell walls unstained.

Note: Alternatively, stages 6, 7 and 8 may be deleted in which case the results will be as follows: parasites not so conspicuously stained violet-purple, with deep purple nuclei. Cellulose walls, yellow to green. Lignified tissue, blue. Tissue nuclei, pale blue with purple nucleoli, and chromosomes deep blue on a purple spindle.

TITAN YELLOW

For the detection of magnesium in plant cells

Solutions required:

A. Titan yellow, special for magnesium test 0·2% aqueous.

B. Sodium hydroxide 10% aqueous.

Technique:

1. Paraffin sections are mounted on slides and brought down to distilled water in the usual manner, or freehand sections of fresh material may be employed.

2. Add one or two drops of the Titan yellow solution, followed by one or two drops of the sodium hydroxide solution.

Results:

If magnesium is present a red coloration is produced.

TRYPAN BLUE

A nuclear stain for plant material

Solutions required:

A. *Formol Acetic alcohol* (*Tellyesniczky*)
Formaldehyde 40% .. .. 5 ml.
70% Alcohol .. .. .. 100 ml.
Glacial acetic acid .. .. .. 5 ml.

B. Trypan blue 1% aqueous .. .. 50 ml.
Ethylene glycol .. .. .. 25 ml.
Absolute alcohol .. .. .. 25 ml.
Zinc sulphate .. .. .. 5 gm.

C. Trypan blue 1% aqueous .. .. 20 ml.
Distilled water .. .. .. 10 ml.
Absolute alcohol .. .. .. 60 ml.
Cresol .. .. 10 ml.
HCl, concentrated .. .. .. 0·1 ml.

D. Exactly the same as solution C but omit the HCl.

E. Absolute alcohol .. .. .. 95 ml.
Cresol .. .. 5 ml.

F. Naphthol yellow, saturated in equal volumes of alcohol and ethylene glycol.

G. Sodium thiocyanate 0·5% in equal volumes of ethylene glycol and absolute alcohol.

H. Ponceau 3R satd. in 80% alcohol 1 volume
80% alcohol .. .. .. 1 volume

Technique:

1. Fix epidermis or smears in solution A.

2. Stain epidermis overnight in solution B or C.

Note: Anther smears are stained in solution D for one half to two hours.

3. Differentiate in 50% alcohol (epidermis for about fifteen minutes; smears for about five minutes), controlling by micro-

scopic examination, observing the nuclei rather than the non-nuclear material, as it may not be possible to remove all the blue stain from the latter without completely decolorizing the nuclei.

Note: If 50% alcohol is found inadequate, then solutions E, F, and G should be tried in turn to ascertain which is the most satisfactory for the particular plant material. Some material, e.g. sweet clover epidermis needs little or no differentiation. Solutions F and G are more active as differentiators than E or 50% alcohol.

4. Place in 95% alcohol for two minutes.

5. Counterstain epidermis if desired with solution H for one minute but this may not be advantageous in the case of smears.

6. Rinse in 95% alcohol.

7. Immerse in two changes of Isopropyl alcohol for two minutes in each.

8. Immerse in a third change of Isopropyl alcohol for five minutes.

9. Clear in xylol and mount in Clearmount or balsam.

Results:

Nuclei, bright blue. If counterstain is used, the background is bright red.

Reference: Hoffmesster, E. R. (1953), *Stain Tech.*, **28**, no. 6, 309.

SECTION 4—CYTOLOGICAL METHODS

ACID FUCHSIN - TOLUIDINE BLUE - AURANTIA

For mitochondria

Solutions required:

A. *Champy's Fluid:*

Potassium dichromate 3%	7 ml.
Chromic acid 1%	7 ml.
Osmic acid 2%	4 ml.

B. Pyroligneous acid — 1 volume
Chromic acid 1% — 2 volumes

C. Potassium dichromate 3%.

D. Acid fuchsin — 10 gm.
Aniline water — 100 ml.

E. Toluidine blue 0·5% aqueous.

F. Aurantia 0·5% in 70% alcohol.

Technique:

1. Tissues are fixed in Champy's Fluid for twenty-four hours; then washed in running water for at least an hour.

2. Immerse in Solution B for twelve to twenty hours; then wash in distilled water for thirty minutes.

3. Mordant in Solution C for three days; then wash in running water for twenty-four hours.

4. Dehydrate; clear; embed in paraffin wax.

5. Sections, 3 to 5μ in thickness are taken down to water in the usual manner; then stained for six minutes by flooding the slide with Solution D, and heating gently till vapour rises.

6. Rinse in distilled water; then counterstain in Solution E.

7. Rinse with distilled water; then stain with Solution F for thirty to fifty seconds.

8. Differentiate with 95% alcohol; dehydrate; clear in xylol and mount.

Results:

Mitochondria are stained red; nuclei, blue. Background, yellow.

ACID FUCHSIN - PICROINDIGO CARMINE

A cytological stain for root tips

Solutions required:

A. Acid Fuchsin 1% aqueous

B. Saturated Picric acid, aqueous .. 1 volume
Saturated Indigo carmine, aqueous 1 volume

C. Acetic acid 0·25% aqueous

Technique:

1. Bring sections through to distilled water in the usual manner.
2. Stain from five to twenty minutes in the acid fuchsin solution.
3. Rinse in water until stain ceases to come out of the sections.
4. Stain from five to fifteen minutes in solution B.
5. Wash with solution C.
6. Wash rapidly in 70% alcohol, in which the sections will appear red.
7. Wash rapidly in 95% alcohol, until sections appear greenish.
8. Dehydrate rapidly with two changes of absolute alcohol.
9. Clear in xylol and mount.

Results:

With onion root tips as an example, chromosomes and late prophase stages are in various shades of bright or dark red. Early prophases, bluish red. Nucleoli, clear blue. Spindle fibres and cell walls are stained a dark blue against a light cytoplasm.

From *Plant Microtechnique* by D. A. Johansen, by courtesy of McCraw-Hill Book Company, Inc., New York.

ALIZARIN RED, S

For mitochondria, cell inclusions, etc. (Benda's method)

Solutions required:

A. Chromic acid 1% .. .. 30 ml.
Osmic acid 2% .. .. .. 8 ml.
Glacial acetic acid .. 6 drops

B. Pyroligneous acid .. .. 100 ml.
Chromic acid 1% .. .. 100 ml.

C. Potass. dichromate 2%.

D. Iron Alum 4% aqueous.

E. Alizarin Red S, saturated in absolute alcohol .. .. .. 1 ml.
Distilled water .. .. .. 90 ml.

F. *Benda's crystal violet:*
Crystal violet, saturated in 70% alcohol .. .. 100 ml.
Alcohol 70% .. .. .. 99 ml.
Hydrochloric acid .. .. 1 ml.
Aniline water .. .. .. 200 ml.

Technique:

1. Fix for eight days in Solution A.

2. Wash for one hour in water; then transfer to Solution B for twenty-four hours.

3. Transfer to Solution C for twenty-four hours.

4. Wash for twenty-four hours in water.

5. Dehydrate; clear; embed in paraffin wax.

6. Mordant sections on slides for twenty-four hours with Solution D; then rinse in water.

7. Stain for twenty-four hours in Solution E.

8. Rinse with water; flood slides with Solution F and warm gently until vapour is given off; or stain at room temperature for twenty-four hours.

9. Rinse in water; then differentiate for two to three minutes in 30% acetic acid until the nuclei appear reddish.

10. Wash in running water for five to ten minutes; blot dry.

11. Dip into absolute alcohol; then pass through bergamot oil into xylol and mount.

Results:

Mitochondria, violet. Chromatin and archoplasm, brownish red. Certain secretion granules, pale violet. Centrosomes, reddish violet.

ANILINE FUCHSIN - IODINE GREEN

For mitochondria

Solutions required:

A. Acid fuchsin aqueous 10% .. 90 ml.
Aniline oil.. .. .. .. 10 ml.
Mix well by shaking at intervals over a period of several hours; then filter.

Note: This solution deteriorates after three or four weeks.

B. Potassium permanganate 1% aqueous.

C. Oxalic acid 5% aqueous.

D. Iodine green 1% aqueous.

Technique:

1. Small pieces of tissue are fixed in Regaud's Fluid for four days.

2. Transfer to 3% aqueous potassium dichromate for eight days changing the solution at intervals of two days.

3. Wash in running water overnight.

4. Dehydrate in alcohol as usual and clear in xylol.

5. Embed in paraffin wax; cut sections not more than 5μ in thickness.

6. Fix sections to slides; pass through xylol and descending

grades of alcohol down to distilled water in the usual manner; then blot carefully.

7. Place the slides, sections facing upwards, on the corner of a tripod; flood the preparation with aniline fuchsin (Solution A) and heat gently with a small bunsen flame until vapour rises.

8. Remove the flame and allow the stain to act for five to ten minutes.

9. Pour off the excess stain and drain for a few seconds.

10. Rinse well in distilled water.

11. Immerse in the potassium permanganate for five seconds; then pour off excess.

12. Immerse in the oxalic acid solution for five seconds; then pour off excess.

13. Wash with distilled water; then stain with the iodine green solution for five to ten seconds.

14. Pour off excess stain; drain for a few seconds; dehydrate quickly with 95% and absolute alcohol.

15. Clear in xylol; mount in Cristalite.

Results:

Mitochondria, crimson. Nuclei, green.

ANILINE FUCHSIN - PICRIC ACID (Altmann)

For mitochondria

Solutions required:

A. *Altmann's Fluid:*

Potassium dichromate 5% aqueous 1 volume
Osmic acid 2% aqueous .. 1 volume

Note: Although the penetration power of this fixative is poor, it is very satisfactory for surface fixation.

B. Acid fuchsin .. .. .. 20 gm.
Aniline water .. .. .. 95 ml.

C. Picric acid saturated in absolute alcohol.

Technique:

1. Small pieces of tissue, not more than 2 mm. in diameter, are fixed for twenty-four hours in Altmann's Fluid.

2. Wash for an hour in running water; then dehydrate; clear, and embed in paraffin wax in the usual manner.

3. Sections, not thicker than 4μ, are brought down to distilled water; then stained for six minutes in Solution B.

4. Pour off excess stain; then blot section carefully; then differentiate and counterstain by flooding the preparation with Solution C.

5. Rinse quickly in 95% alcohol; then dehydrate with absolute alcohol; clear in xylol, and mount.

Results:

Mitochondria are stained crimson against a vivid yellow background.

ANILINE SAFRANIN (Babe's)

For demonstrating mitosis in animal tissues

Solutions required:

A. Aniline Safranin (Babe).

B. Alcohol 95% .. .. .. 99 ml.
Acetic acid 1% .. .. .. 1 ml.

Technique:

1. Fix small pieces of tissue in Flemming's or Hermann's fluid and embed in paraffin wax.

2. Stain sections for five to ten minutes in the safranin solution.

3. Rinse in water.

4. Differentiate in the acid alcohol (Solution B).

5. Rinse in 95% alcohol.

6. Rinse in absolute alcohol.

7. Clear in xylol and mount.

Results:

Mitotic figures are stained an intense red, while resting nuclei are deep pink to colourless.

BASIC FUCHSIN - PICRO INDIGOCARMINE (Alcoholic)

For plant tissues, as a cytological stain for root tips

Solutions required:

A. Basic fuchsin 1% in 70% alcohol.

B. Indigocarmine .. .. .. 0·6 gm.
Distilled water .. .. .. 50 ml.
Picric acid, saturated aqueous 50 ml.

Technique:

1. Sections are brought down to distilled water; then stained from ten to twenty minutes in the basic fuchsin solution.

2. Rinse in distilled water until the stain ceases to come out.

3. Stain from five to fifteen minutes in Solution B.

4. Rinse quickly in 70% alcohol until the sections appear red to the naked eye.

5. Rinse rapidly in 95% alcohol, followed by absolute alcohol until the preparation appears green to the naked eye.

6. Clear in xylol; then mount.

Results:

Chromosomes and late prophase stages, varying shades of red; early prophases, bluish red; nucleoli, clear blue; spindle fibres and cell walls, dark blue; cytoplasm, light blue.

BASIC FUCHSIN - PICRO INDIGOCARMINE (Aqueous)

For chromosomes in plant tissues

Solutions required:

A. Basic fuchsin 1% aqueous.

B. Picro indigocarmine.

Technique:

1. Material is fixed in Nevashin or Regaud.

2. Wash in running water; dehydrate, clear and embed in paraffin wax in the usual manner.

3. Fix sections to slides and remove paraffin wax with xylol.

4. Pass through absolute 90% and 70% alcohol down to distilled water, as usual.

5. Stain in the basic fuchsin solution for about five minutes.

6. Rinse in distilled water.

7. Stain in the picro indigocarmine solution for ten to twenty minutes.

8. Rinse in distilled water to which a few drops of hydrochloric acid have been added.

9. Differentiate in 80% alcohol for half to one minute, until red coloration ceases to come away.

10. Dehydrate with 70%, 90% and absolute alcohol.

11. Clear in xylol, and mount.

Results:

Chromosomes, brilliant red. Nucleoli and other cell components, sky blue.

BREINL'S TRIPLE STAIN

For chromosomes

Solutions required:

A. Iodine .. .. 1 gm.
Potassium iodide .. .. .. 2 gm.
Alcohol 90% .. .. .. 100 ml.

B. Safranin 1% in 50% alcohol

C. Methylene Blue, polychrome (Unna)

D. Orange tannin

Technique:

1. Fix sections to slides and remove paraffin wax with xylol as usual.

2. Rinse with two changes of absolute alcohol.

3. Mordant by immersing in solution A for fifteen minutes.

4. Rinse well with water.

5. Stain for at least half an hour in the safranin solution in a closed jar or tube.

6. Wash well with water.

7. Stain with solution C for ten minutes.

8. Wash with water; drain and remove excess water, but do not allow the preparation to dry.

9. Place the slide under the microscope; then cover the preparation with orange tannin, by means of a pipette.

10. Observe the progress of the staining under the microscope until the orange tannin has replaced the blue in the cytoplasm.

11. Withdraw excess fluid from the slide by means of a piece of filter paper to avoid the risk of spilling over the microscope stage and condenser.

12. Take the slide away from the microscope and wash well with 95% alcohol.

13. Dehydrate in absolute alcohol.

14. Clear and complete differentiation in aniline oil.

15. Rinse with cedarwood oil.

16. Drain and remove excess cedarwood oil from around the preparation by blotting or wiping with a clean fluffless duster.

17. Mount in Cristalite or Canada balsam in Xylol or Emexel mountant.

Results:

Chromosome threads, blue. Metaphase chromosomes, red. Cytoplasm, pale yellow.

Reference: Bolles-Lee, 11th edition, p. 664.

COPPER CHROME HAEMATOXYLIN (Bensley)

For mitochondria

Solutions required:

A. Altmann's Fluid

B. Copper acetate, saturated aqueous

C. Haematoxylin 10% in absolute alcohol
ripened .. .. 5 ml.
Distilled water .. .. .. 90 ml.

D. Potassium chromate neutral, 5% aqueous

E. *Weigert's Borax Ferricyanide Mixture*

Borax 1% aqueous	..	..	100 ml.
Potass. ferricyanide	..	..	1·25 gm.

Technique:

1. Fix very small pieces of tissue in Altmann's fluid from 12 to 24 hours.

2. Wash, dehydrate, clear and embed in paraffin wax in the usual way.

3. Cut sections from 4 to 5μ in thickness and affix them to slides.

4. Remove paraffin wax with xylol.

5. Wash well with absolute alcohol.

6. Wash with 90% alcohol.

7. Wash with 70% alcohol.

8. Wash with distilled water.

9. Immerse in solution B for 5 minutes.

10. Wash with several changes of distilled water for a total time of one minute.

11. Stain with the haematoxylin (solution C) for 1 minute.

12. Wash in distilled water.

13. Immerse in solution D for one minute, which should turn the sections dark blue-black: if they are only light blue in colour, rinse in distilled water and return to the copper acetate solution and if necessary repeat several times until the sections are dark blue-black after a minute in solution D, or until no increase in colour is obtained.

14. Rinse with distilled water.

15. Immerse in solution B again for a few seconds; then again rinse in distilled water.

16. Differentiate under the microscope with a mixture consisting of one volume of solution E and two volumes of distilled water.

17. Wash with tap water for 6–8 hours.

18. Dehydrate, clear and mount.

Results:

Mitochondria are stained deep blue against a yellowish background.

COTTON RED - METHYL VIOLET - ORANGE G

A cytological stain for plant tissues

Solutions required:

A. Cotton red 1% aqueous.

B. Methyl violet 10B 1% aqueous.

C. Orange G - Clove oil.

Technique:

1. Stain sections of Flemming-fixed material in the cotton red solution from eight to twenty-four hours.

2. Wash well in water; then stain in the methyl-violet solution from ten minutes to an hour according to the material.

3. Pour off excess stain; then wash in water.

4. Wash well with 95% alcohol; followed by thorough rinsing with absolute alcohol.

5. Drain well, and blot carefully.

6. Immerse in orange G - clove oil from five to ten seconds.

7. Pour off excess stain; then wash the preparation with clove oil.

8. Differentiate in a fresh lot of clove oil for about one half to one minute, controlling by examination under the microscope until the violet stain is satisfactory.

9. Pour off excess clove oil; then wash with three changes of xylol.

10. Mount in Canada balsam in xylol.

Results:

Metaphase and anaphase chromatin, red. Prophase chromatin, violet. Chromoneata, red. Chromosome matrix, purple. Spindle fibres and plastids, violet. Cytoplasm, light brownish grey. Nucleoli, red.

FEULGEN STAIN

For mitosis in plant cells

Solutions required:

A. Feulgen's fuchsin.

B. Potass. metabisulphite 10% .. 5 ml.
N/1 HCl .. .. .. 5 ml.
Distilled water .. .. .. 100 ml.

C. Fast green FCF 0·5% in 70% alcohol.

Technique:

Tissues should be fixed in Flemming and embedded in paraffin wax.

1. Sections are brought down to distilled water.

2. Rinse in N/1 HCl; then transfer to N/1 HCl at 60° C. for four to five minutes; afterwards rinsing in cold N/1 HCl.

3. Rinse in distilled water; then stain for three to four hours in Solution A.

4. Drain and immediately transfer to a stoppered jar containing Solution B for ten minutes; then transfer to a second jar of Solution B for ten minutes, followed by ten minutes in a third jar.

Note: The jars must be kept closed.

5. Rinse in distilled water; then counterstain for one half to one minute in Solution C.

6. Dehydrate; clear and mount.

Results:

Chromosomes, reddish violet.

GENTIAN VIOLET - PICRIC ACID - IODINE

For chromosomes in plant cells

Solutions required:

A. Potassium iodide .. .. .. 0·5 gm.
Iodine, resublimed .. .. .. 0·5 gm.
Water .. .. 5 ml.
Absolute alcohol .. .. .. 45 ml.

Dissolve the potassium iodide in the water, then add the iodine and shake till dissolved, afterwards adding the alcohol and mixing well.

B. Crystal violet 1% aqueous.

C. Picric acid 0·5% in absolute alcohol.

Technique:

1. Fix in Navashin or Fleming.
2. Take the preparation (smear or section) through to 70% alcohol in the usual way.
3. Pass through 95% alcohol.
4. Mordant for fifteen minutes in solution A.
5. Rinse well in water.
6. Stain with solution B for ten to fifteen minutes.
7. Rinse well in water.
8. Immerse again in solution A for a few minutes.
9. Rinse in 95% alcohol.
10. Immerse the preparation for about one second in the picric acid solution.
11. Wash immediately with absolute alcohol for a few seconds.
12. Rinse in clove oil until the violet stain ceases to come out of the preparation.
13. Wash well in two changes of xylol.
14. Immerse in a jar of xylol for about an hour.
15. Drain off excess xylol and mount in Canada balsam in xylol or in Cristalite.

Results:

Chromosomes: rich purple, each chromosome being sharply defined, Cytoplasm: yellow.

Note: This technique is an improvement on Newton's Gentian Violet - Iodine in that fading does not occur and sharp differentiation is obtained of Chromosomes that are close together.

Reference: Smith, F. H. (1934), *Stain Tech.*, 9 95–6.

HAEMATOXYLIN (Regaud)

For mitochondria

Solutions required:

A. Formalin (40% formaldehyde) .. 25 ml.
Potassium dichromate .. .. 3 gm.
Distilled water .. .. .. 98 ml.

B. Potassium dichromate 2·5% aqueous.

C. Ammonia - ferric alum 10% aqueous.

D. Haematoxylin (Regaud).

E. Iron Alum 5% aqueous.

Technique:

1. Small pieces of tissue are fixed for three to five days in Solution A, which should be freshly prepared and changed each day.

2. Mordant by immersing for ten to fourteen days in Solution B.

3. Wash in running water for twenty-four hours.

4. Dehydrate by immersing in ascending grade of alcohol, and clear in the usual manner.

5. Embed in paraffin wax and cut sections no thicker than 5μ.

6. Fix sections to slides; de-wax and pass through descending grades of alcohol down to water.

7. Mordant sections for one to three days in a stoppered jar in the incubator at 37° C.

8. Rinse for five minutes or so in running water.

9. Immerse for twenty-four hours in the haematoxylin (Solution D).

10. Differentiate in Solution E, controlling at intervals by examination under the microscope.

11. Rinse in running tap water for twenty to thirty minutes.

12. Drain off excess water; then rinse with 95% alcohol.

13. Dehydrate with absolute alcohol.

14. Clear in xylol and mount.

Results:

Mitochondria are conspicuously stained intense black.

HAEMATOXYLIN - SAFRANIN

For differentiating nucleoli and other nuclear constituents in plant cells

Solutions required:

A. Iron Alum 3%

B. Haematoxylin 10% in absolute alcohol, well ripened .. .. .. 5 ml.
Distilled water .. .. .. 45 ml.

C. Safranin saturated in absolute alcohol .. .. 1 volume
Aniline water .. .. .. 1 volume

D. Picric acid 1% in 95% alcohol.

Technique:

1. Fix sections to slide and take down to distilled water.
2. Mordant with solution A for two to three hours.
3. Wash in running water for five minutes.
4. Rinse in distilled water.
5. Immerse in the haematoxylin for an equal length of time.
6. Differentiate very carefully with solution A, controlling by examination under the microscope while the preparations are still wet.
7. When the nucleoli have been almost completely decolorized remove the Iron alum solution quickly by rinsing in water.
8. Wash in running water for an hour or longer.
9. Immerse in solution D for twelve to sixteen hours.
10. Differentiate for a few seconds in Picro-alcohol (solution D.)
11. Wash with two changes of absolute alcohol.

12. Clear in xylol.

13. Mount in Cristalite or Canada balsam in xylol and examine under the oil immersion objective.

Results:

Nucleoli, brilliant red. Chromosomes during metaphase and anaphase are stained a bright red and stand out sharply against a dark background; during prophase and telophase the chromosomes are considerably darker. Trabants are usually red and are attached to the chromosomes by a black thread.

Note: This technique gives the best results after a killing fluid containing picric acid, but chromic acid fixatives also give good results.

Reference: *Plant Microtechnique* (1st ed., p. 75) by D. A. Johansen, by courtesy of the McGraw-Hill Book Co.

IRON ACETO CARMINE (Belling)

For chromosomes in microsporocytes

Solutions required:

Iron Aceto Carmine (*Belling*)

Acetic acid 50% aqueous	100 ml.
Carmine, powdered	1 gm.

Boil under reflux condenser for half an hour.
Cool and filter.
Add a few drops of ferric hydrate in 50% acetic acid.

Technique:

Temporary preparations:

1. Anther smears are made on slides by teasing and squashing.

2. Place a drop of the stain on a smear; then cover with a coverslip.

3. Place slide, coverslip facing upwards over a corner of a tripod and heat gently until steam rises from the edges of the coverslip.

4. Examine under the microscope at once.

Results:

Chromatin is stained deep translucent red, while cytoplasm is unstained.

Permanent preparations:

5. Proceeding from Stage 4 (above), immerse the preparation in a Petri dish containing 10% acetic acid until the coverslip becomes loose.

6. Immerse the slide and the coverslip in a mixture consisting of equal volumes of absolute alcohol and glacial acetic acid, contained in another Petri dish.

7. Transfer to a dish containing a mixture consisting of three volumes of absolute alcohol and one volume of glacial acetic acid.

8. Transfer to a mixture consisting of one volume of glacial acetic acid and nine volumes of absolute alcohol.

9. Transfer to absolute alcohol; then into two changes of xylol.

10. Refix the coverslip to the slide with a drop of Cristalite.

METHYL GREEN - ACID FUCHSIN - ERYTHROSIN

A cytological stain for plant cells

Solutions required:

A. Methyl Green 1% aqueous.

B. Acid fuchsin 1% aqueous.

C. Erythrosin 1% aqueous.

Technique:

1. Take sections down to distilled water in the usual manner.

2. Stain for an hour in solution A.

3. Remove excess stain by washing thoroughly with water.

4. Stain in the acid fuchsin solution for one minute.

5. Wash well with distilled water to remove excess stain.

6. Stain with the erythrosin solution for two or three seconds.

7. Wash well with distilled water and drain and blot off excess water, but do not allow the section to dry.

8. Rinse with 70% alcohol.

9. Rinse quickly with 90% alcohol.

10. Dehydrate quickly but thoroughly with two changes of absolute alcohol.

11. Clear in xylol and mount in Canada balsam in xylol or in Emexel.

Results:

Chromatin granules and nucleoli in early stages of microsporogenesis are stained green while the linin threads are red. Chromosomes are stained brilliant green in later stages.

Reference: Cooper, D. C. (1931) *American J. Bot.*, **18**, 337.

METHYL GREEN - ACID FUCHSIN

For chromosomes, etc., in plant tissue

Solutions required:

A. Methyl green 2% aqueous .. 100 ml.
Acetic acid 1% .. .. .. 2 ml.

B. Acid fuchsin 1% aqueous.

Technique:

1. Sections are brought down to water; then stained from six to seven hours in the methyl green solution.

2. Wash in water until the stain is almost entirely removed from the non-lignified elements (this process should be controlled by examination under the microscope while the preparation is still wet).

3. Rinse in water.

4. Stain from five to ten minutes in the acid fuchsin solution, controlling under the microscope to ensure that the green is not extracted from the lignified tissues.

5. Wash rapidly in 95% alcohol, followed by absolute alcohol.

6. Clear in clove oil; then pass through xylol and mount.

Results:

Cytoplasm and plastin, light red; chromosomes and nuclei, green.

METHYL VIOLET - EOSIN SCARLET

A botanical stain for mitosis in root tips

Solutions required:

A. Methyl Violet 5B aqueous 1%.

B. Picric acid 1% in absolute alcohol.

C. Eosin scarlet, saturated in equal volumes of clove oil and absolute alcohol.

Technique:

1. Sections are fixed to slides and taken down to water in the usual manner.

2. Stain for twenty to thirty minutes in the methyl violet solution; then wash with distilled water.

3. Differentiate with the picric acid solution for a few seconds.

4. Immerse in absolute alcohol to which has been added 0·15% strong ammonia solution, for fifteen to twenty seconds.

5. Immerse in absolute alcohol for ten to twenty seconds.

6. Counterstain for five to fifteen seconds in the eosin scarlet solution.

7. Clear in clove oil; rinse with xylol; mount.

Results:

Resting and dividing chromatin, purple. Cytoplasm, pink. Cell walls, red. Plastin, deep red.

NIGROSINE

For the study of salivary chromosomes of *Drosphila*

Solutions required:

A. Acetic acid 45% aqueous

B. Absolute alcohol .. .. .. 70 ml.
Distilled water .. .. .. 29 ml.
Hydrochloric acid, pure, conc. .. 1 ml.

C. Nigrosine, alcoholic (Michrome brand) .. .. 1 gm.
70% alcohol .. .. .. 100 ml.

Technique:

1. Dissect the specimen in 45% acetic acid.

2. Treat with solution B for one to two minutes, keeping glands well covered with the reagent.

3. Wash the specimen carefully two or three times with distilled water, after which a large drop of water is left on the specimen for five minutes.

4. Drain thoroughly and carefully.

5. Add a drop of the nigrosine solution.

6. Place a coverslip over the preparation but do not squash.

7. Allow the stain to act for ten to fifteen minutes.

8. Place a piece of blotting paper over the coverslip and slide and squash the preparation.

9. Immediately place a large drop of glycerine on one edge of the coverslip.

10. The preparation may now be examined at once, but after about twenty-four hours the glycerine will have penetrated the specimen thoroughly, when it is recommended, for the sake of convenience, to remove the excess liquid and ring the coverslip with wax.

Note: Preparations obtained as above will last for reasonably long periods provided they are carefully handled.

Results:

Salivary glands specifically and intensely stained; only the outer bands being coloured, except in heterochromatin regions. When the preparation is ageing, however, the interbands as well as the cytoplasm, become faintly stained.

Reference: Pares, Ramon (1953), *Nature*, **172**, no. 4390.

SAFRANIN CRYSTAL VIOLET (Hermann)

For chromosomes

Solution required:

A. Safranin .. .. 1 gm.
Aniline water .. .. .. 50 ml.
Absolute alcohol .. .. .. 50 ml.

B. Crystal violet 1% in 70% alcohol.

Technique:

1. Stain for three to twenty-four hours in the safranin solution in a closed jar or tube.
2. Destain briefly with 50% alcohol.
3. Counterstain with the crystal violet from thirty seconds to five minutes.
4. Rinse quickly with 90% followed by absolute alcohol.
5. Clear with clove oil.
6. Wash with xylol, and mount.

Results:

Chromosomes and nucleoli are stained red; resting nuclei and chromatin granules violet: propase and early telophase nuclei may show violet chromonema and red chromatin granules; cytoplasm, light violet; spindle, deeper violet.

Reference: Hermann, *Arch. Mik. Anat.*, **34**, 58.

SAFRANIN - GENTIAN VIOLET - ORANGE G.

(Flemming Tricolour stain)

For chromosomes, etc.

Solutions required:

A. Safranin 3·5% in 95% alcohol .. 10 ml.
Aniline water .. .. .. 10 ml.

B. Gentian violet 5% aqueous.

C. Orange G 1% aqueous.

Technique:

1. Sections of material, which have previously been fixed in Flemming's fixative, are stained for two to twenty-four hours with the safranin solution, in a stoppered staining jar.

2. Differentiate in acid alcohol until colour almost ceases to come out of the sections and microscopic examination shows the chromosomes in metaphase stained clearly with the safranin.

3. Rinse with distilled water.

4. Immerse in the gentian violet solution, in a staining jar for one half to three hours, washing with water at intervals and examining under the microscope until the prophase chromatin is clearly stained violet.

5. Differentiate in the orange G solution for thirty to sixty seconds when pale violet clouds should be given off.

6. Rinse in absolute alcohol until scarcely any more colour comes out of the sections.

7. Differentiate in clove oil and while very faint clouds of colour are still coming away, rinse with benzol and mount in balsam.

Results:

Chromosomes, red. Nucleoli, red. Metachromatic chromatin, deep purple. Cytoplasm, orange. Spindle, blue-violet.

Note: The action of this stain should be so precise that in one strand of chromatin the diffused portion will take the violet, while the condensed section should be stained with the safranin.

WRIGHT'S STAIN

For chloroplasts in tissue spreads and for plant cytology

Note: Valuable for showing cytoplasmic changes in various tissues and cell inclusions. Reveals cytoplasmic differentiations and experimental change. The method is generally applicable for cytological work wherever material may be spread (not smeared) and dried rapidly. Produces excellent preparations of chloroplasts. Where cells separate well and where there is neither an excess of water in tissue fluid nor concentrated protein scum, conditions are favourable for a good spread of uninjured cells and an excellent stain.

Solution required:

Wright's stain.

Technique:

1. A Nitella cell, carefully isolated from the plant, is placed on a piece of filter paper from which it is transferred to a clean dry slide.

2. The uninjured cell is pricked so that it bursts out its sap and the chloroplasts in effective spread.

3. Lift the deflated cell with needles and transfer to an adjacent dry region and tear to release more chloroplasts with a small amount of sap. Arrange areas of the cell wall smoothly so that no portion of the cell or its contents are lost in the preparation. By spreading the cell fluid about in this way there is no excess in any region.

4. Dry rapidly with gentle heat.

5. Place 1 ml. of Wright's stain on the dried spread, and leave it to act for one minute; then add 2 ml. distilled water and rock the slide gently to ensure thorough mixing.

6. Allow this diluted stain to act for three to five minutes; then pour off and wash with distilled water until the thin portion of the films appears pink to the naked eye; then pour off excess stain.

7. Wash with distilled water and allow the preparation to dry before examining.

Results:

Chloroplasts of normal Nitella are basophilic and of marked alveolar structure. Large, homogeneous-appearing vacuole plastids which are noted in the streaming of the living cell vacuole are brilliantly eosinophilic. However, they prove to be complex with a central raphe, usually colourless arrangements of both basophilic and eosinophilic granules; and both bilaterally distributed lacunae varying according to experimental conditions from colourless to brilliant blue. The staining of Tunicate material is so critical that species differences are readily noted in comparative studies of the same cells.

Reference: Koehring, Vera (1951), *Stain Tech.*, **26**, 29.

SECTION 5—
FLUORESCENCE MICROSCOPY

(a) GENERAL INFORMATION

FLUORESCENCE is the property possessed by many substances of converting short wavelengths of light into longer wavelengths. In microscopy the substances and structures of most interest are those which convert ultraviolet light into light of the visible spectrum, as only substances of this character can be observed directly. It is, of course, well known that most living organisms are profoundly affected by short light waves, and a great deal of information as to their structure has been obtained by the study of the appearance of these organisms under the influence of invisible light rays. If individual cells or structural units are examined before, during, and after ultraviolet treatment, enough of this effect should be discovered to impart some understanding as to the changes which occur in the animal, or plant, as a whole.

In fluorescence microscopy structural details are rendered visible by: (*a*) *innate auto-fluorescence*, a property possessed by most tissues which when excited by short light waves become clearly visible since they become luminous and glow or " fluoresce " with a radiance of their own, or by (*b*) *secondary fluorescence* which is known as " fluorochromy " and is brought about by the process of treatment of the tissues with fluorescent dyes and certain alkaloids (e.g. Berberine sulphate) and other substances.

It is proposed to deal only with fluorochromy in the short space of this chapter.

Fluorescent dyes and other substances used for this purpose are known collectively as " fluorochromes "; these materials are selectively absorbed by certain parts of the cell. When tissues, bacteria or protozoa, which have undergone treatment with fluorochromes are examined under the microscope, using ultraviolet light instead of transmitted light of the visible spectrum, they become visible as bright luminous objects against a dark background. Cells stained with fluorochromes absorb the ultraviolet rays of short wavelength, and emit this energy in the form of

fluorescent light in the visible spectrum. Basic and acidic fluorochromes act specifically to stain certain cellular structures as do the more common microscopic stains such as, for instance, methylene blue and eosin. The colour and the intensity of fluorescence depends on the relative basophilia and acidophilia of the individual cells, and upon the nature of the particular fluorochrome.

Fluorochromy may be employed with advantage in the study of living organisms: for instance, uranin, a non-toxic stain, may be injected into mice and frogs and the living organs can be studied without interfering with their functioning. Fluorochromy is also of practical importance for the demonstration of diphtheria bacilli, tubercle and leprobacilli, malaria, etc., as well as for virus research.

(b) EQUIPMENT REQUIRED

Contrary to the generally held belief, the apparatus required for fluorescence microscopy is fairly simple and inexpensive. A special microscope is not required.

1. B.T.H. Mazda Mercury Vapour Lamp (box type) ME 250 w/50/5.

2. A simple convex lens to project the image of the light source through a suitable blue filter to the microscope mirror. The lens and light source should be encased with a black hood to prevent scattering of the rays.

3. A yellow filter which is placed in the oculars of the microscope to prevent any harmful effect of ultraviolet light to the microscopist's eyes. For this purpose Ilford's minus blue Micro 4 is recommended.

It is, of course, essential that fluorescence microscopical examinations must be carried out in a darkroom to be successful. It has been stated that microscope slides of special glass are necessary for fluorescence microscopy, but provided they are not more than 1·3 mm. in thickness ordinary microscopic slides have been found quite satisfactory.

(c) STAINING METHODS

Notes on fluorochromic staining technique:

(*a*) Fixatives containing salts of heavy metals (with the exception of zinc); chlorine, bromine, iodine, and nitro compounds should be avoided as these exert a quenching effect on fluorescence. The most suitable fixatives are 5 to 10% formalin, or Kahle's fluid.

(*b*) If tissues are embedded in paraffin wax, all traces of the wax, which is auto-fluorescent, must be removed before sections are stained and examined.

(*c*) A special grade of immersion oil known as fluoroil, which is non-fluorescent, should be used for high-power examination, since cedarwood oil and most of the immersion oils available for ordinary microscopy are unsuitable for fluorescence work.

(*d*) The usual mounting media, as used for ordinary microscopy, contain highly fluorescent materials which render them unsuitable for fluorescence work, and should, therefore, be avoided. For temporary mounts, glycerine may be used, and for permanent mounts there is a satisfactory medium on the market under the name of *Fluormount*.

Fluorochromes, of which auramine O, coriphosphine, acridine yellow H107, aesculine, acridine orange, primuline, thiazole yellow are examples, are frequently used in very dilute solutions to produce characteristic fluorescent colours, and when preparations which have been treated with these solutions are examined in transmitted light of the visible spectrum, they appear to be unstained or only very faintly tinted.

Some explanation as to the colour differentiation obtained by the use of general-purpose fluorochrome of which acridine yellow H107 is an example, is explained by the fact that fluorescence colour is effected by hydrogen-ion concentration, and as fluorochromes also exhibit differential absorption by various tissues, the production of a great variety of fluorescent colours is brought about by the influence of these two factors. Nuclei can be differentiated from cytoplasm by the use of any one of the following general fluorochromes: acridine yellow H107, coriphosphine, phosphine 3R, or acriflavine.

Titan yellow, rhodamine B, phosphine 3R, methylene blue are

all excellent fluorochromes for fat, while berberine sulphate is much used for protozoal parasites, particularly for malaria. Thioflavine has been found satisfactory for virus and for bacteria, as well as for general fluorochromic staining, while acriflavine has been used for trypanosomes and as a general stain, and uranin is one of the most suitable fluorochromes for intravital staining. All these fluorochromes are used in very dilute aqueous solutions, that is to say, something to the order of 0·1 to 0·01 per cent.

1. Differential Staining of Cytoplasm, Nuclei, Nucleoli and Chromatin

Solutions required:

A. Congo red 0·1% aqueous or acid fuchsin 0·1% aqueous.

B. Acridine yellow H107 0·1% aqueous or coriphosphine 0·1% aqueous.

Technique:

1. Sections are stained for two minutes with the Congo red or acid fuchsin solution.

2. Pour off excess Congo red; then stain for two minutes with the acridine yellow or coriphosphine solution.

This technique gives a very sharp differentiation.

2. Staining of Fat

Method A. *Solution required:*

Phosphine 3R 0·1% aqueous, or methylene blue 1% aqueous.

Technique:

1. Stain frozen section for two minutes; then rinse in water.

2. Examine in water.

Results:

With phosphine 3R fat is observed as a silver fluorescence

against a brown background and due to the omission of lipid solvents in this technique, smaller and more numerous fat droplets can be seen than in the case of the sudan techniques as used in ordinary microscopy. With methylene blue fat gives a blue fluorescence.

Method B. *Solution required:*

Thioflavine S 1% aqueous.

Technique:

1. Frozen sections are stained for two minutes; then rinsed in water.

2. Examine in water.

Results:

Fats appear violet against a dark background.

Note: With this method fewer lipids are stained than with phosphine 3R.

3. Intravital Staining with Fluorochromes

Dyes used for this purpose must be water soluble, non-diffusible in the living body, non-toxic in the workable dilutions required, and highly fluorescent even in greatly diluted solutions. Uranin possesses all these qualifications and is one of the most useful fluorescent dyes for intravital work, as stated earlier in this chapter. Acriflavine is another useful dye for this purpose, although it is not so intensely fluorescent as uranin. The fluorescence of uranin is impaired in basic solution so that it appears most readily in organs of an acid reaction. It is used in 0·1% solution in physiological saline, in which form it should be injected into the animal's blood stream or into the organ to be studied. The colour of its fluorescence varies with hydrogen-ion changes and consequently it is of great value as an intravital hydrogen-ion indicator. The colour changes are easily visible in dilutions to the order of one part in ten millions and in dilute solutions the intensity of the fluorescence has a definite relation to the concentration of the dye and consequently the intensity of the fluorescence

serves as an indicator of the amount of uranin present. Primulin, thioflavine, rhodamine B, berberine sulphate have also been found useful for intravital work.

Many important discoveries in the biological field are due to fluorescence microscopy and although a great many beautiful and highly contrasting colour combinations have been obtained by the techniques devised up to the present time, fluorescence microscopy has scarcely yet emerged from the embryonic stage, and there is tremendous scope for experiment and improvement both in regard to technique and apparatus.

4. Staining of Muscle

Solutions required:

A. Primulin 0·1% aqueous.

B. Sodium nitrite 2% aqueous.

C. Hydrochloric acid, conc. .. 10 ml.
Distilled water .. .. .. 90 ml.

D. Resorcinol, saturated aqueous solution.

Technique:

1. Sections are stained for two to five minutes with primulin solution.

2. Wash quickly; then immediately transfer to the sodium nitrite to which has previously been added, with stirring, an equal volume of the diluted hydrochloric acid (Solution C above), and leave therein for fifteen to twenty minutes.

3. Wash in water; then immerse for fifteen to thirty seconds in the resorcinol solution.

Results:

Muscle tissue fluoresces with a strikingly luminous green fluorescence.

5. Differentiation of Nerve Tissues

Method A. *Solution required:*

Acridine yellow H107 aqueous 0·1%.

Technique:

1. Sections are stained with the acridine yellow for two minutes.
2. Pour off excess stain and rinse with distilled water.
3. Dehydrate in the usual manner; clear in xylol; mount in Fluormount.

Results:

Neurophile nerve tissue appears light blue, while cortical nerve tissue is light yellow to orange.

Method B. *Solution required:*

Acridine orange 0·1% aqueous.

Technique:

1. Sections are stained with the acridine orange solution for two minutes.
2. Pour off excess stain, rinse with distilled water.
3. Dehydrate as usual, clear in xylol in Fluormount.

Results:

Neurophile is bluish-grey while cortical tissue is brownish orange.

Method C. *Solution required:*

Acriflavine 0·1% aqueous.

Technique:

1. Stain with the acriflavine solution for two minutes; then pour off excess and rinse in water.
2. Dehydrate rapidly; clear in xylol and mount in Fluormount.

Results:

Neurophile is blue, while cortical tissue appears brownish yellow.

6. Staining and counting of bacteria, and differentiating living from dead cells, in soil

Solutions required:

A.	Buffer solution pH 7·5	200 ml.
B.	Solution A	100 ml.
	Acridine Orange, FS	0·1 gm.
C.	Solution A	5 ml.
	Solution B	5 ml.
D.	Solution A	5 ml.
	Solution B	10 ml.
E.	Solution A	3 ml.
	Solution B	9 ml.
F.	Solution A	2 ml.
	Solution B	8 ml.
G.	Solution A	1 ml.
	Solution B	9 ml.

Technique:

1. Place 1 gm. of the sifted soil in each of six test tubes, and label them consecutively B, C, D, E, F, and G.

2. Add 10 ml. of the appropriate solution to the five tubes labelled B, C, D, E, and F (i.e. 10 ml. solution B to tube B; 10 ml. solution C to tube C, etc.).

Note: As soils differ in their ability to absorb the stain, the same soil must be stained with different concentrations of the acridine orange to ascertain the most suitable concentration. Solution G will be used for differentiating between living and dead bacteria.

3. Shake each of the five tubes thoroughly; then leave them to stand for five or ten minutes.

4. The most suitable concentration for the particular soil is that contained in the tube, which shows the least amount, compared with the others, of excess dye, and this specimen should be used for examination as follows:

(*a*) *For qualitative analysis of autochthonic bacteria:*

The dyed soil suspension is strongly centrifuged; the upper (liquid) layer is poured off and discarded, leaving a small quantity

of sediment which is then well mixed with a drop of paraffin oil and covered with a coverslip before examination with the oil immersion lens.

(*a*) *For bacterial counts:*

A loopful of the stained and shaken soil suspension is placed on a slide, covered with a coverslip, and examined with an oil immersion lens. A counter of 20μ depth combined with a counting ruled eyepiece is used. The particles of soil covered with humus and the particles of humus slime show a dim red fluorescence and the living bacteria are green. The stained bacteria are not killed and may be used for culturing.

To differentiate between living and dead cells, solution G should be used: living cytoplasm and nuclei show green fluorescence, whilst dead protoplasm develops a bright copper-coloured fluorescence.

Reference: Strugger, S. (1948), *Canad. J. Res. Sec. C*, **26**, pp. 188–93.

7. Vital Staining of Living Trypanosomes in Blood (S. Strugger's Method)

Solution required:

Acridine orange 0·01% in 0·85% sodium chloride.

Technique:

1. A drop of blood suspected of containing trypanosomes is mixed on a slide with a few drops of the acridine orange solution, and covered with a coverslip.

2. Examine with a blue light fluorescence microscope, which may be constructed as follows: parallel rays produced by attaching a convex lens to the lamp are filtered with a curvette (2½ cm. thick) filled with saturated solution of copper oxide ammonia so that only the blue light reaches the plane mirror of the microscope. A filter containing an orange glass which absorbs blue light quantitatively, but allows green, yellow and red to pass unchanged, is placed over the ocular. A slide with powdered anthracene in liquid paraffin is used for focusing.

Results:

Trypanosomes and leucocytes are seen as motile, bright, green luminous bodies while the erythrocytes are scarcely visible and are non-fluorescent.

8. Staining of Trypanosomes in Dried Blood Films

Solution required:

Auramine O 0·1 % aqueous ..	100 ml.
Phenol crystals	5 gm.

Technique:

1. Fix air-dried blood films, suspected of containing trypanosomes, in pure methyl alcohol for two to five minutes, then wash with distilled water.

2. Stain for about four minutes in the auramine solution then wash with distilled water for one to two minutes, keeping the slide in the dark.

Results:

Examined with blue light fluorescence microscope, erythrocytes appear as dark green, slightly luminous circles, while trypanosomes shine with a bright golden fluorescence against a black background. By covering the film with a drop of liquid paraffin and a coverglass the contrast will be even more marked.

9. Staining of Tubercle Bacilli

Solutions required:

A. Auramine O aqueous 0·1% .. 100 ml.
Phenol crystals .. .. .. 5 gm.
Dissolve by shaking. Do not apply heat as auramine decomposes at 40° C.

B. Methylene blue 0·1% aqueous.

C. Potassium permanganate 0·1% aqueous.

Technique:

Place sandalwood oil or fluoroil between the slide and condenser. Three sides of the microscope should be enclosed with a shield to exclude extraneous light.

Sections:

1. Fix tissues in 5 to 10% formalin for twelve to twenty-four hours; embed in paraffin wax and cut sections 5 to 10μ in thickness.

2. De-wax as usual, taking care that all traces of wax are completely and finally removed from sections.

3. Bring sections down to distilled water as usual; then flood with the auramine solution and warm (not over 40° C.) for five to ten minutes, afterwards washing with distilled water.

4. Counterstain in methylene blue solution for thirty seconds; then rinse in distilled water, dehydrate as usual; clear in xylol and mount in Fluormount.

Smears:

1. Stain for five minutes in the auramine solution, afterwards washing with tap water and decolorizing in 25% sulphuric acid.

2. Immerse in the potassium permanganate solution for about thirty seconds to overcome any interfering fluorescence.

Results:

Tubercle bacilli appear as thin shining, slightly curved rods against a dark background.

An Improved Method of Staining Tubercle Bacilli (H. Lempert's Method)

Solutions required:

A. Phenol 3% in distilled water .. 100 ml.
Auramine O .. .. .. 0·3 gm.
Dissolve by shaking. Do not apply heat, as auramine decomposes when heated above 40° C.

B. Hydrochloride acid, pure .. 0·5 ml.
Sodium chloride .. .. 0·5 gm.
Methyl alcohol, pure .. .. 75 ml.
Distilled water .. .. .. 25 ml.

C. Potassium permanganate 0·1% aqueous.

Technique:

1. Smears of sputum or centrifuged urine deposits are fixed by heat in the usual manner.

2. Immerse the smears in the auramine solution for eight to ten minutes, afterwards washing them with tap water.

3. Decolorize by immersing for two minutes in each of two changes of Solution B; then wash in tap water.

4. Immerse in the potassium permanganate solution for about thirty seconds; then wash with tap water; blot and dry.

Note:

It is claimed that this method gives more positives than the Ziehl-Neelsen technique and there is a saving in time. Using Lempert's method, tubercle bacilli are visible under the two-thirds or the quarter-inch objective with a ×8 eyepiece, and the one-sixth-inch objective may be used for confirmation.

10. Staining Virus with Primulin

Solution required:

Primulin 0·1% aqueous.

Technique:

1. Stain for thirty seconds.

2. Mount if desired in a medium consisting of 100 gm. of best pale gum acacia dissolved in 100 ml. water and 50 ml. glycerine with the addition of 10 gm. chloral hydrate.

Note: With primulin, which may also be used as a general fluorochrome, a blue-violet fluorescence is obtained.

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SECTION 6—
HISTOCHEMICAL METHODS

MICRO - INCINERATION

Micro-incineration is a technique employed to ascertain the relative positions occupied by inorganic salts in fixed tissues, and the total distribution of these mineral substances in cells and tissues.

There are two methods of fixation, neither of which is perfect, namely; (*a*) Freeze-dry and (*b*) Chemical fixation. The first method requires reagents and apparatus which are not normally available in the average laboratory; also the method is exceedingly laborious and time consuming: there is a certain element of risk of injury to the operator who may not be accustomed to handling such substances as liquid nitrogen at a temperature of —170°C. Moreover, it is extremely doubtful if the elaborate "Freeze-Dry" method offers any more accurate results than those obtained by the relatively simple chemical fixation method.

By employing either method the total mineral distribution cannot be determined with an error of less than, say, 10% as all minerals will not survive the incineration processes, and it is, therefore, proposed to describe here the more simple and straightforward chemical method of fixation which can be carried out in any laboratory.

Solution required:

Absolute alcohol	90 ml.
Formaldehyde 40%	10 ml.

Note: It is best to use freshly distilled alcohol. On no account must the alcohol have been dried with copper sulphate nor any other mineral reagents. The formaldehyde must not have been treated with calcium carbonate, buffer salts or any other mineral reagents. It is essential that all the reagents used must be free from, and remain free from, dust and mineral matter; this refers also to the xylol and the paraffin wax. All aparatus used must be scrupulously clean, washed out with several lots of distilled water, partially dried with absolute alcohol, and in all cases, where at all possible, finally dried with a clean dust-free cloth.

1. Fix small pieces of tissue in absolute alcohol, with a mechanical agitator, for twelve to twenty-four hours, changing the alcohol at intervals of one hour during the day.

2. Clear in freshly filtered xylol.

3. Infiltrate with and embed in wax, which must not be plunged into water to hasten cooling.

4. Clean slides, in readiness to take the sections, by washing them several times in distilled water, then partially drying them with absolute alcohol, and finally drying them with a dust-free cloth. Store the slides away from dust and mineral matter until they are required for use.

5. Cut serial sections 3 to 5μ in thickness, taking particular care at this stage that they are not contaminated with dust or mineral matter.

6. Press serial sections onto slides, without any fixative or spreading agent, with the fingers which have been washed and dried with absolute alcohol.

Note: It is now, at this stage, more than ever necessary to guard against contamination by dust.

7. Mount alternate serial sections on albuminized slides for normal histological staining and comparison later with the incinerated specimen.

8. The slides bearing the sections for incineration are placed in a quartz-tube electric furnace, but if that is not available a muffle furnace, the inside of which is entirely free from dust and debris, will serve the purpose.

9. Gradually raise the temperature so that it reaches a maximum of 60° C. at the end of the first three minutes.

10. During the next three minutes, gradually raise the temperature to 70° C.

11. Raise to 80° C. during the next two minutes.

12. Raise to 200° C. during the next five minutes.

13. During the next twenty-five minutes raise the temperature at the rate of 90° C. per five minutes, when the maximum of 650 ° C. will be attained.

14. Turn off the heat and allow the slides to cool in the oven for about thirty minutes.

15. Take the slides out of the furnace and lay coverslips over the sections, with a pair of forceps.

16. Seal the coverslips with De Kotchinsky's cement.

17. De-wax and stain the control serial sections, which have been set aside for the purpose, with suitable stains.

18. Examine the unstained, incinerated specimens comparing them with control serial sections. This can be most conveniently accomplished by employing two identical microscopes connected with a comparing ocular, the microscope with the normally stained sections being illuminated with ordinary bright-light condenser, and the one with the incinerated sections with a dark-field illuminator: this method facilitates the location of the same structure in both types of sections.

Notes:

There are many methods, some of which are given in this book (*see* index) of identifying the numerous minerals which might be present, but it is not possible to give them all here without entering the realms of Mineralogy, Fluorescence Analysis and kindred subjects which are foreign to a book of this kind, but which are well catered for in other text-books to which the reader is referred as well as to the various references given in *McLung's Handbook of Microscopical Technique*, *Laboratory Technique in Biology and Medicine*, by E. V. Cowdry, and the methods given in *Microscopic Histochemistry* by G. Gomori.

3-HYDROXY - 2-NAPHTHOIC ACID - TETRAZOTIZED o-ANISIDINE

For demonstrating sites of carbonyl activity in frozen sections

Solutions required:

Important: All alcohol used in this technique must be aldehyde free.

A.	3-hydroxy - 2-naphthionic acid hydrazide	0·1 gm.
	50% alcohol	95 ml.
	Acetic acid glacial 5%	5 ml.

B.	Absolute alcohol	30 ml.
	Phosphate buffer 1/15 M (pH 7·2)..	10 ml.
	Distilled water	20 ml.
	Tetrazotized o-anisidine	0·06 gm.

Technique:

1. Tissues are fixed in 10% formalin (or 5% formol saline).

2. Without washing out fixative, cut frozen sections and place them on slides.

3. Allow the sections to become affixed to slides by drying in the atmosphere; then wash out formalin with several changes of water.

4. Immerse in solution B for one or more hours in an incubator at room temperature.

5. Remove excess reagent (solution B) by washing for two hours in 50% alcohol (aldehyde free).

6. Wash in several changes of water for several hours.

7. Immerse in solution C for one to two minutes.

8. Wash in several changes of distilled water to which a few drops of acetic acid have been added.

9. Drain off excess liquid; then mount in Glycerin Jelly.

Results:

Sites of carbonyl activity are indicated by the production of a blue pigment.

Reference: Seligman, A. M. and Ashbel, R. (1951), *Cancer*, **4**, 579–96.

METANIL YELLOW - IRON HAEMATOXYLIN

For radioautographs prepared by mounting tissue sections directly onto photographic emulsions for study of thyroid carcinoma and all organs of man, as well as laboratory animals to which radio isotopes have been administered

Solutions required:

A. Metanil Yellow 0·25% aqueous.

B. Haematoxylin .. .. .. 1 gm.
Ethyl alcohol 95% .. .. .. 100 ml.

C. Ferric chloride hydrated 50%
aqueous .. .. 5 ml.
Distilled water .. .. .. 95 ml.

D. Solution B .. .. 1 volume
Solution C .. .. 1 volume
Mix well, and allow to stand for two or three weeks before use. **Filter immediately before use.**

Note: The fresh solution must not be used as it darkens the emulsion gelatine; 60 *ml. of this solution is sufficient for about twenty radioautographs.*

E. Acetone and Xylol, equal volumes

Technique:

1. Surgical and post-mortem specimens from patients having recently received radioiodine (I^{131}) and tissues from laboratory animals to which radioiodine, radiosulphur (S^{35}) or radiophosphorus (P^{32}) has been administered, are fixed in Bouin's fluid.

2. Embed in paraffin wax, using the Dioxan technique.

3. Cut sections 7 to 10μ in thickness.

4. Transfer the section ribbons to the darkroom, place in a waterbath, and float them onto a photographic plate. (X-ray film, Kodak Medium lantern slides or Kodak nuclear track plates, are suitable for this purpose.)

5. Allow the sections to dry on the plate, when they will become permanently attached to the photographic emulsion.

Note: For each isotype the processing fluids should be examined by a Geiger counter to ensure that there is no significant loss of the radioactive material from the tissue.

6. After proper photographic exposure, removal of the paraffin wax, development, fixation, washing and drying, the autograph is stained as follows:

7. Stain with metanil yellow (solution A) for five to fifteen seconds.

8. Wash in tap water until the slide is pale yellow: this should take about fifteen seconds.

9. Stain in solution D for one to three minutes.

10. Rinse with three changes of 70% alcohol for five seconds in each.

11. Wash for five seconds in 95% alcohol

12. Wash with acetone for five seconds.

13. Wash with solution E (acetone-xylol); for five seconds.

14. Wash with three changes of xylol.

15. Mount synthetic resins; e.g. D.P.X. or Cristalite.

Results:

Colloid, cytoplasm and connective tissue elements are stained from light yellow to light brown. Cell nuclei are stained deep-blue to black.

Sites of radioactivity are indicated by blackening of the photographic emulsion.

Reference: Simmel, Eva B., Fitzgerald, P. J. and Godwin, J. T. (1951), *Stain Tech.*, **26**, 25–8.

METHYL - GREEN - PYRONIN - RIBONUCLEASE (Brachet)

For detecting ribonucleic acid and desoxyribonucleic acid in the same cell

Solutions required:

1. Zenker's fixative containing 5% acetic acid

or

Carnoy Fixative

or

Serra Fixative:

Absolute alcohol	60 ml.
Formalin (40% formaldehyde) ..	30 ml.
Acetic Acid, glacial	10 ml.

2.* *Methyl green-Pyronin* (*Brachet*)

Methyl green, OO chloroform washed	0·15 gm.
Pyronin G	0·25 gm.
Alcohol 95%	2·5 ml.
Acetate Buffer pH 4·7	97·5 ml.

Methyl green, OO is washed repeatedly with chloroform, to extract all traces of methyl violet. The washed methyl green is then dried, and 0·15 gm. is weighed out and dissolved in the acetate buffer and the alcohol with the pyronin.

Note: the stain is liable to deteriorate after two or three weeks.

* See note (*d*) at the end of this technique.

3. †Ribonuclease 0·1% in distilled water which has been adjusted to pH 6·0

† If this is not available a suitable ribonuclease extract can be prepared in the laboratory as follows:

I. Pass 0·5 to 1 kilo Ox pancreas through a meat mincer.
II. Pound it to a smooth pulp with a mortar and pestle.
III. Suspend the pulp in one or two volumes of N/10 acetic acid for sixteen to twenty-four hours.
IV. Boil for ten minutes.
V. Cool; then filter.
VI. Adjust the pH of the filtrate to pH 6·0.
VII. Filter.
VIII. Add a crystal of thymol or camphor, as a preservative and store in the refrigerator: under these conditions the solution will keep for several months.

Technique:

Note: It is not possible to give absolute rules concerning the following procedure, as the methods of fixation, the solubility of the ribonucleic acid of the organ to be studied, and the activity of the ribonuclease, are all possible variables. The technique given below, which should be regarded as a basis for experiment, may be varied to suit individual lines of research and investigation.

1. Fresh material should be used and objects or slices of tissue, which must not be more than 2–3 mm. in thickness, are fixed for a maximum of one hour in one of the above fixatives.

2*a*. After Zenker, wash with tap water for twenty-four hours.

2*b*. After Carnoy or Serra, wash in two or three changes of 95% alcohol for the minimum time necessary to remove the fixative.

3. Dehydrate and embed at once, through alcohol and toluol, in paraffin wax.

4. Fix sections alternatively to slides marked A, B and C.

5. Remove paraffin wax with toluol, and pass through the usual descending grades of alcohol to distilled water.

6. Stain slide A immediately with the methyl green-pyronin for twenty minutes.

7. Meanwhile place slide B in distilled water, adjusted to pH 6·0 in the oven and leave there for an hour at 37° C.

8. Place slide C in the ribonuclease solution in the oven at 37° C. and leave there for an hour.

9. Meanwhile take slide A, after it has been in the methyl green-pyronin for twenty minutes, and wash it rapidly with distilled water.

10. Slide A is then differentiated with 95% alcohol for five to ten minutes.

11. Dehydrate slide A with absolute alcohol; then wash with toluol and mount in balsam or D.P.X. or Cristalite.

12. When slide B has been in the oven for an hour, stain it with the methyl green-pyronin, differentiate, dehydrate and mount exactly as in the case of slide A.

13. When slide C has been in the oven for an hour, stain, differentiate, dehydrate and mount exactly as slides A and B.

14. Examine and compare specimen C with A and B.

Notes:

(*a*) Methyl green is unique in that it will stain some, but not all basophilic substances. It will stain high-polymer DNA as it occurs in the nuclei, but it will not stain depolymerized DNA or RNA in any form. In specimen B (above) it will be found that although the methyl green staining may have been completely abolished, Feulgen's reaction remains unchanged. Interesting comparisons can be made between sets of slides as B and C (above) with sections from the pancreas, kidney, lung, liver, genital glands, skin, etc. (See Professor J. Brachet's original 1942 paper*).

(*b*) It may be found necessary, depending upon the fixative used and the organ to be studied, to raise the concentration of the ribonuclease solution to as much as 0·6% to obtain optimum results.

(*c*) The quality of the dyes, methyl green and pyronin, is a factor of great importance if good results are to be obtained with this technique.

(*d*) In Professor Brachet's original paper (1942) the Unna Pappenheim Methyl Green-pyronin, formula as below, was employed:

Methyl green, OO	0·15 gm.
Pyronin	0·25 gm.
Alcohol 96%	2·5 ml.
Glycerin	20 ml.
Phenol 0·5% aqueous	100 ml.

This stain requires very rapid differentiation with the 95% alcohol as the stain, particularly the pyronin is liable to be washed out completely: differentiation here requires a great deal of practice and skill. For this reason Professor Brachet (1953) recommends the modified formula. However, chloroform washed methyl green is somewhat troublesome to prepare, and many workers may prefer to use the original Unna Pappenheim Methyl Green-Pyronin.

Results:

Specimen A:

The presence of ribonucleic acid (of the nucleoli and of the cytoplasm) is indicated as red particles, and the desoxyribonucleic acid (of the chromatin) of the same cell, as blue.

Specimen B:

Treatment with hot water abolishes the methyl green staining of desoxyribonucleic (while Feulgen reaction remains unchanged). The absence of the methyl green staining confirms the presence and localization of desoxyribonucleic acid.

Specimen C:

Ribonuclease brings about a complete loss of basophilia due to ribonucleic acid, not basophilia due to mucopolysaccharides. The absence of the red granules indicates the presence and localization of ribonucleic acid. The Feulgen reaction of the nuclei is unchanged.

References:

* Brachet, J. (1942), *Archives de Biol.*, **53**, 207–57. " Localisation des acides pentosenucleiques dans les tissues animaux et les œufs d'Amphibiens en voie de développement. "

Brachet, J. (1953), *Q. J. Micr. Sc.*, **94**, 1–10. " The use of basic dyes and ribonuclease for the cytochemical detection of ribonucleic acid. "

NILE BLUE SULPHATE - SAFRANIN

An histochemical technique for demonstrating phospholipids in frozen sections

Solutions required:

A. Formalin 10% .. .. .. 100 ml.
Calcium Chloride 1% .. .. 1 ml.
Calcium Carbonate .. .. .. 2 gm.
Shake well: filter before use.

B. Safranin 1% aqueous .. .. 100 ml.
Aniline Oil .. .. 2 drops

C. Nile blue sulphate, saturated aqueous 100 ml.
Sulphuric acid 0·5% .. .. 10 ml.
Boil for 2 hours under reflux condenser.
Filter before use.

D. Acetic acid 5% aqueous.

E. HCl conc. .. .. 0·5 ml.
Distilled water .. .. .. 99·5 ml.

Technique:

1. Fix material in solution A: then cut frozen sections, without embedding: or the material may be embedded, if desired, in gelatine or carbowax.

Note: Frozen sections should not be stored in water for more than ten to fifteen minutes.

2. Stain in the safranin solution for five minutes.

3. Rinse in distilled water.

4. Stain in the nile blue sulphate for ninety minutes at 60° C.

5. Rinse in distilled water.

6. Immersé in acetone heated to 50° C. on a water bath.

7. Remove the acetone from the source of heat and allow the sections to remain in it for half an hour.

8. Differentiate in 5% acetic acid for thirty minutes.
9. Rinse thoroughly in distilled water.
10. Refine the differentiation in the 0·5% HCl (Solution D).
11. Wash in several changes of distilled water.
12. Mount in glycerine jelly.

Results:

Phospholipids, blue. Nuclei, red.

Reference: Menschik, Z. (1953), *Stain Tech.*, **28**, 13–18.

PHOSPHOMOLYBDIC ACID - EOSIN

For the histochemical localization of choline-containing lipids, in frozen sections

Solutions required:

A. Acetone .. .. 1 volume
Ether .. .. 1 volume
B. Chloroform .. .. 1 volume
Absolute alcohol .. .. .. 1 volume
C. Phosphomolybdic acid.
1% in solution B.
D. Stannous chloride 1%.
in 3N hydrochloric acid.
E. Eosin 1% aqueous.

Technique:

1. Dip frozen sections into acetone-ether.
2. Immerse in the phosphomolybdic acid solution for fifteen minutes.
3. Rinse in solution B.
4. Dip into solution D.
5. Counterstain with the eosin solution for one to two minutes.
6. Mount in glycerine jelly.

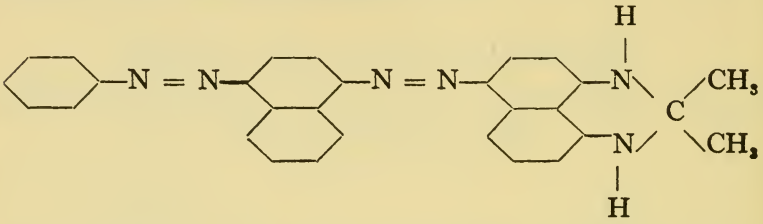
Results:

Positive areas are stained blue, whilst negative areas are red.

Reference: Landing, B. H. (1952), Uzman, L. L. and Whipple, Ann. *Lab. Invest.*, **1**, 456–2.

SUDAN BLACK

The use of Sudan Black, also known as Sudan Black B, has been described in various works on Histochemistry. As far as I am aware, the correct structural formula of the Sudan Black molecule has not yet been disclosed in any literature published in any country, although it appears that this information has frequently been sought after by medical and biological research workers. It may, therefore, be of interest and help to research workers to give the formula here:



The molecular weight and other information regarding Sudan Black will be found on page 435.

SECTION 7—SMEAR PREPARATIONS

ALBERT'S STAIN

(Laybourn's modification)

For diphtheria bacilli

Solutions required:

A.

Toluidine Blue	0·15 gm.
Malachite Green	0·2 gm.
95% alcohol	2 ml.
Glacial acetic acid	1 ml.
Distilled water	100 ml.

Dissolve by warming or shaking well; allow to stand for twenty-four hours; then filter.

B.

Potassium iodide	3 gm.
Iodine	2 gm.
Distilled water	300 ml.

Dissolve the potass. iodide in about 5 ml. of the distilled water; add the iodine and shake until dissolved; then add the remainder of the distilled water and shake well.

Technique:

1. Fix films by gentle heat; then stain for five minutes with Solution A.
2. Without washing apply Solution B for one minute.
3. Wash rapidly with water; blot dry.

Results:

Granules of diphtheria bacilli are stained bluish-black; bars of bacilli dark green to black. Body of cells, light green; other organisms, light green.

Note: Best results are obtained with a young culture (eighteen to twenty-four hours old) on serum medium.

ALCIAN BLUE

For bacterial polysaccharides and capsules

Note: This dye will stain bacterial capsules and insoluble polysaccharides in both pure and mixed cultures of bacteria and protozoa. It is specific for bacterial polysaccharides, and enzymatic degradation of these results in the loss of this stain.

Solutions required:

A. Alcian blue .. .. 1 gm.
Alcohol 95% .. .. .. 100 ml.

B. Solution A .. .. 1 volume
Distilled water .. .. .. 9 volumes
Note: This solution which deteriorates within a few days, should only be prepared as and when required for immediate use.

C. Carbol fuchsin (Ziehl-Neelsen)

Technique:

1. Fix air-dried smears by flaming in the usual way.

2. Stain with solution B for one minute.

3. Pour off excess stain and wash with water; then allow the preparation to dry in the air.

4. Counterstain with carbol fuchsin for a few seconds.

5. Wash immediately with distilled water to prevent overstaining.

6. Allow the preparation to dry in the air; then examine.

Results:

Capsule and other bacterial polysaccharides are stained blue: cellular material, red.

Note: Various carbohydrates including adonitol, arabinose, cellobiose, dextrin, glucose, inositol, inulin, mannitol, mannose, rhamnose, sorbitol, trehalose, xylose, and certain enzymes including papain, pepsin, rennin gave a positive reaction with alcian blue.

The fact that the internal polysaccharides of the cell remain unstained is attributed to the complexity of the dye molecule which prevents its penetration of the cell wall.

Reference: McKinney, Ross E. (1953), *J. Bact.*, (U.S.A.), **66**, 453–4, "Staining bacterial polysaccharides."

ANILINE BLUE - EOSIN B

A simple and rapid technique for spermatozoa, which is particularly suitable for dog and human semen

Solutions required:

A.	Ether	1 volume
	Absolute alcohol	1 volume
B.	Aniline blue aqueous	2 gm.
	Eosin, B	1 gm.
	Phenol 1% aqueous	20 ml.
	Distilled water	60 ml.

Technique:

1. Fresh semen is allowed to stand for about one half to one hour until it liquifies.

2. Prepare thin even smears of the liquified semen on scrupulously clean and dry slides or coverslips.

3. Fix for 3 minutes in a mixture consisting of equal volumes of ether and absolute alcohol; then allow to dry in the air.

4. Flood smears with solution B and warm over a steam bath or hot plate while the stain is allowed to act for 5–7 minutes at 40–60° C.

5. Pour off excess stain.

6. Wash the preparation thoroughly with distilled water.

7. Drain and allow to dry thoroughly in air.

8. Mount in a neutral synthetic resin such as D.P.X., Clearmount or Cristallite.

Results:

Sperm structure	*Stain reaction*
Galea capitis	Pale bluish-grey; sharply outlined
Cell membrane	Bluish-grey; sharply outlined
Nuclear membrane (shell)	Bluish-grey; sharply outlined
Acrosome	Slate blue
Nucleas	Pink
Neck	Sharply outlined (dark blue), inside colourless

End knobs	Dark blue
Middle piece	Sheath dark blue, centre dark pink
Axial filament	Dark blue
Tail	Dark blue

Notes:

It is suggested that the technique which has been tried out only on dog and human semen, might be applied to other species.

The technique gives good differentiation and preservation of cytological structure, reliable fixation and staining and undistorted and easily recognizable detail, upon which assays of semen for fertility depend.

There is a tendency for abnormal forms of spermatozoa to stain more intensely than the normal forms.

Reference: Casarett, George W. (1953), *Stain Tech.*, **28**, no. 3, 125–7.

ANILINE GENTIAN VIOLET

A simple and rapid stain for *Treponema pallida*

Solutions required:

A. Aniline gentian violet.

B. Sodium hydroxide 1% aqueous 50 ml.
Absolute alcohol .. .. 1·5 ml.

C. Alcohol 5%.

Technique:

1. A loopful of serum from the lesion is spread into a film on a slide and dried in air. If an enlarged syphilitic gland is to be examined, 0·5 ml. sterile saline should be injected into the gland; then aspirated with a 1-ml. syringe with a 22-gauge needle. Haemoglobin may be removed, if necessary, with distilled water.

2. Stain with ten drops each of Solutions A, B and C for two minutes, rocking the slide to ensure thorough mixing.

3. Wash in running tap water for twenty seconds; dry and examine.

Results:

Treponema pallida, deep purple, with distinct regular spirals free from precipitate. *Spirochaeta refringens* and other spirochaetes, purplish black, with regular open and coarse spirals.

AZUR L

For the detection and staining of epidermophytic infection

Solution required:

Azur L 0·5% aqueous.

Technique:

1. Scrapings from around the margin of non-purulent areas of infected skin or from the tops of vesicles are transferred to a slide.

2. Treat three to ten minutes, depending on the size and thickness of the specimen, with Carnoy's fluid; then pour off carefully so that any floating debris is carried away and the fungus is left adhering to the slide.

3. Dry over a flame, taking care that the remaining Carnoy's fluid does not take fire.

4. Stain two or three minutes with 0·5% aqueous Azur L.

5. Rinse by adding distilled water drop by drop, taking care to avoid washing away the specimen.

6. Drain well; then dry cautiously over a flame and mount.

Results:

Fungal filaments, dark blue against a light blue background.

BASIC FUCHSIN

For *Treponema pallida* in smears

Solutions required:

A. Potassium hydroxide 1% aqueous.

B. Basic fuchsin 10% in absolute alcohol .. .. 5 ml.
Distilled water .. .. .. 95 ml.

Technique:

1. Fresh, air-dried smears are fixed for five minutes in pure methyl alcohol.

2. Allow the smears to dry in air; then add a few drops of 1% potassium hydroxide aqueous solution followed immediately by twice the number of drops of basic fuchsin solution.

3. Rock the slide to ensure thorough mixing of the two solutions; then leave until the liquid becomes decolorized, which should take about three minutes.

4. Pour off excess and rinse the preparation thoroughly with distilled water.

5. Drain, and blot dry carefully but thoroughly.

6. If it is desired to preserve the preparation it should be mounted at once in Cristalite.

Results:

Treponema pallidum, scarlet. Background, pink.

BREED'S STAIN

For staining and counting bacteria in milk

Solution required:

Methylene Blue	0·3 gm.
95% alcohol	30 ml.
Distilled water	100 ml.
Phenol	2½ gm.

Shake well until dissolved.

Technique:

1. Mark off 1 sq. cm. on a piece of white paper and superimpose a slide.

2. Place 0·01 ml. of the milk to be tested on the slide and spread, by means of a needle, into a film exactly 1 sq. cm., as indicated by the marking on the paper.

3. Dry on a level surface, by heating gently.

4. Immerse in xylol for a few minutes to remove the fat; drain well; wash with absolute alcohol; then 95% alcohol; immerse in 90% alcohol for a few minutes.

5. Stain for two minutes with Breed's stain, prepared as above.

6. Wash quickly in 90–95% alcohol until the intense blue colour changes to a faint tinge.

7. Dry and examine.

Results:

Bacteria are stained dark blue against a lighter blue background.

BRILLIANT CRESYL BLUE

For reticulated cells and platelets

Solutions required:

A. Brilliant Cresyl Blue 0·3% in pure absolute ethyl alcohol.

B. Leishman stain or Wright's stain.

Technique:

1. Place a drop of 0·3% Brilliant Cresyl Blue stain in absolute alcohol on a slide and allow it to dry.

2. A drop of blood 2 to 3 mm. in diameter is placed on another slide and brought in contact with the dried stain; the two slides are then manipulated hinge-like until all the stain has gone into solution and the blood appears blue-black. Allow the slides to come into contact to spread the drop; then separate the slides and allow the films to dry.

3. Counterstain with Leishman or Wright by the standard technique.

Results:

Reticulum of immature red cells is stained clear cut blue; background, pale blue (fresh), or pink. Blood platelets, pale blue or lilac.

Note: The counterstain may be omitted if it is desired only to count the platelets.

The number of red cells per cm. should be determined separately in a haemocytometer, and the ratio of platelets to red cells computed from the stained preparation.

For reticulum

Stock solution:

Brilliant Cresyl Blue	1½ gm.
Normal saline (0·85% NaCl) ..	100 ml.

Technique:

1. Mix a small quantity of the stock solution of the stain with 140 times its volume of normal saline solution.

2. Mix the blood in a white-cell counting pipette in the proportion of 1 volume blood to 20 vols. of the diluted staining solution. Shake the mixture for five minutes in the pipette, and place in a blood counting cell.

3. The fresh preparations are sealed with Vaseline to prevent drying, and are counted immediately. At least 1,000 should be counted for each test.

Result:

Reticulum only stained (blue).

CARBOL CRYSTAL VIOLET

For fibrin network in blood smears

Solution required:

Carbol Crystal Violet

Technique:

Note: To retain the normal arrangement of the filaments in the fibrin network, the slides must be slowly and carefully placed in, and taken out of the reagents.

1. Fresh thick blood smears are made on scrupulously clean slides and placed in a moist chamber, immediately, to prevent drying.

Note: The moist chamber may consist of a petri dish the lid of which has two sheets of filter paper, moistened with warm water to accelerate clotting.

2. After the blood has coagulated and the fibrin framework has formed (this takes about six to ten minutes) take the slides out of

the moist chamber and transfer to distilled water to haemolyse the red corpuscles: this takes about five to thirty minutes.

3. Change the water when it is tinged red.

4. When the haemolysis is complete: that is when all traces of red colour has disappeared from the slides and the fibrin network appears as a whitish film on the slide, remove slides and carefully blot away excess water, but do not blot network owing to risk of displacement.

5. Stain for eight minutes in carbol crystal violet.

6. Rinse in distilled water and carefully blot off excess water.

7. Pass through three or four changes of dioxane and mount quickly in D.P.X. or Cristallite or Clearmount, before the network drys.

Results:

Fibrin network is stained violet. White cells appear as irregularly shaped black dots. Platelets appear as black dots about the size of a pin head.

Reference: Badertscher, J. A. (1952), *Stain Tech.*, **27**, no. 4, 217–20.

CARBOL FUCHSIN

For *Treponema pallida* and other spirochaetes

Solutions required:

A. Potassium permanganate aqueous.	5%
B. Carbol fuchsin (Ziehl Neelsen) ..	1 volume
Distilled water	9 volumes

Technique:

1. Air-dried smears are fixed in methyl alcohol for five minutes.

2. Immerse for three minutes in the potassium permanganate solution.

3. Wash with distilled water.

4. Immerse in the diluted carbol fuchsin solution for two minutes.

5. Rinse with distilled water.

6. Drain; dry thoroughly but carefully.

7. If it is desired to preserve the preparation, mount at once in Cristalite.

Results:

Treponema pallidum, light reddish brown. Mouth spirochaetes and *Treponema refringens*, deep reddish brown.

CARBOL FUCHSIN

For acid-fast bacteria, in sputum, including tubercle bacilli

Solutions required:

A. Ziehl Neelsen carbol fuchsin.

B. *Acid alcohol:*

Alcohol 95%	97 ml.
Conc. Hydrochloric acid ..	3 ml.

C. Loeffler Methylene Blue.

Technique:

1. Sputum smears are fixed on a flat surface over boiling water.

2. Stain with Solution A for three to five minutes, heating the slide till steam rises.

3. Pour off excess and allow the slide to cool somewhat before rinsing in water.

4. Decolorize in Solution B until only a faint pink colour comes away; then wash with water.

5. Counterstain with Solution C for ten to thirty seconds; then wash in water; dry and examine.

Results:

Acid-fast bacteria, red. Other organisms, blue. Background, faint blue or colourless.

CARBOL FUCHSIN - BORREL BLUE

For leprosy and for tubercle bacilli

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Sulphuric acid 5%.

C. Hydrochloric acid .. .. 1 ml.
Alcohol 70% .. .. .. 99 ml.

D. Borrel Blue .. .. .. 5 ml.
Distilled water .. .. .. 20 ml.

Technique:

1. Air-dried smears are fixed in absolute alcohol for three minutes.

2. Place the preparation on the corner of a tripod; flood with carbol fuchsin; then heat gently with a small bunsen flame until steam rises.

3. Allow the stain to act for five minutes.

4. Decolorize with 5% sulphuric acid until only a faint pink colour comes away.

5. Wash in 60% alcohol until no more stain comes out.

6. Rinse in distilled water.

7. Counterstain in the diluted Borrel Blue for one half to one minute.

8. Pour off excess stain; rinse with distilled water; drain and blot dry.

Results:

Bacillus leprae and tubercle bacilli bright red. Other bacteria are stained blue, while cells, cell debris and mucin in sputum are in varying shades of blue.

Note: In the case of leprosy, differentiation of the carbol fuchsin (Stages 4 and 5) must be very carefully carried out, as this organism is more easily completely decolorized than tubercle bacilli.

CARBOL FUCHSIN - BRILLIANT YELLOW

For tubercle bacilla in sputum smears

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Brilliant yellow 1% aqueous .. 15 ml.
Sulphuric acid 25% .. .. 40 ml.
Distilled water .. .. .. 30 ml.
Absolute alcohol .. .. 10 ml.

Technique:

1. Fix sputum smears on a horizontal surface over boiling water

2. Stain with carbol fuchsin solution for three to five minutes, heating over a small bunsen flame in the usual manner, until steam rises.

3. Pour off excess stain.

4. Allow the slide to cool somewhat before rinsing the preparation thoroughly with distilled water.

5. Counterstain and differentiate for one half to one minute with the brilliant yellow solution.

6. Wash well with distilled water.

7. Drain; blot thoroughly, and examine.

Results:

Tubercle bacilli are stained red against a clear yellow background.

CASTANEDA'S STAIN

For Rickettsiae and elementary bodies

Solutions required:

A. Phosphate buffer solution pH 7·5.. 20 ml.
Formalin (Formaldehyde 40%) .. 1 ml.
Methylene blue 1% in absolute methyl of ethyl alcohol .. .. 0·15 ml.

B. Safranin O aqueous 0·2% .. .. 1 volume
Acetic acid 0·1% .. .. .. 3 volumes

Technique:

1. Spread smears of infected tissue on scrupulously clean slides; then allow to dry.

2. Stain with solution A for three minutes.

3. Pour off excess stain; then without washing counterstain for one to three seconds in the safranin solution.

4. Wash in running water.

5. Drain and dry thoroughly, and examine.

Results:

Rickettsiae, blue; protoplasm and nuclei of the cells, red.

CRYSTAL VIOLET

For bacterial capsules

Solutions required:

A. Crystal violet 0·5%.

B. Copper sulphate crystals .. 5 gm.
Distilled water .. .. .. 25 ml.

Technique:

1. Stain air-dried smears for three to four minutes in the crystal violet solution.

2. Rinse well in the copper sulphate solution.

3. Blot dry, and examine.

Results:

Capsules are stained light blue, while the bacterial cells are dark blue.

CRYSTAL VIOLET

A simple stain for spirochaetes

Solutions required:

A. Potass. permanganate 1% aqueous.

B. Crystal Violet 2% aqueous.

Technique:

1. Air-dried films are fixed by flaming.

2. Immerse in Solution A for ten minutes; then wash with distilled water.

3. Stain for eight to ten minutes in Solution B.

4. Wash in distilled water; dry and examine.

Results:

Spirochaetes, bluish black and well defined.

Note: For *Treponema pallida*, best results are obtained by warming Solution A on the slide.

DAHLIA

For the rapid staining of Heinz bodies in erythrocytes in blood smears of animals poisoned by certain chemicals such as aniline, phenylhydrazine, and its derivatives

Solutions required:

A.	Dahlia	0·2 gm.
	Ethyl alcohol 95%	100 ml.

Technique:

1. Moderately thick blood smears are made on scrupulously clean slides, then allowed to dry in air.

2. Cover the freshly dried smears with the staining solution and allow it to act for thirty seconds.

3. Pour off excess stain and wash well with running water to remove the excess dye.

4. Drain and blot dry; examine under the oil immersion objective.

Results:

Heinz bodies are visible as blue granules, the depth of colour depending somewhat on their size and the thickness of the smear.

Reference: Webster, S. H., Liljegren, E. J. L. and Zimmer, D. J. (1948), *Stain Tech.*, **23**, 97.

DORNER'S SPORE STAIN

Solutions required:

A. Carbol fuchsin (Ziehl Neelsen).

B. Nigrosin, water soluble .. .. 10 gm.
Distilled water .. .. .. 100 ml.
Dissolve by heating, cool, filter and add:
Formalin .. .. 0·5 ml.

Technique:

1. A heavy suspension of the culture is made in a test-tube with three drops of distilled water and three drops of carbol fuchsin are then added. Heat on a boiling water bath for ten to fifteen minutes.

2. A loopful each of the stained preparation and Solution B are mixed on a slide, spread into a thin smear and dried rapidly; then examined.

Results:

Spores, red. Bodies of bacteria, almost colourless against a dark grey background.

EHRLICH'S TRIACID STAIN

For blood smears

Solution required:

Ehrlich's triacid stain.

Technique:

1. Air-dried films on slides or on coverglasses are fixed by heat.
2. Allow to cool for a few seconds, then flood films with the staining solution and allow the stain to act for five to ten minutes.
3. Rinse with distilled water until the stain ceases to come out.
4. Drain off excess water.
5. Blot and dry thoroughly; mount in Cristalite.

Results:

Erythrocytes, orange. Eosinophile granules, bright red. Basophile granules, unstained. Neutrophile granules, purple. Lymphocytes, unstained. Nuclei, pale green.

EOSIN - GENTIAN VIOLET

For basal bodies of cilia

Solutions required:

A. Haematoxylin (Ehrlich).

B. Eosin 0·5% aqueous.

C. Aniline gentian violet (Stirling).

D. Lugol's iodine solution.

E. Aniline oil .. .. 10 ml.
Xylol .. .. 20 ml.

Technique:

1. Stain for two minutes in the haematoxylin solution.
2. Blue and wash in tap water or in lithium carbonate solution.
3. Stain for one minute in the eosin solution.
4. Wash with water.
5. Stain for two hours in the aniline gentian violet.
6. Wash well in tap water.
7. Stain in Lugol's iodine for ten to fifteen minutes.

8. Wash well with tap water; drain and blot dry.

9. Differentiate in Solution E then wash in several changes of xylol and mount.

Results:

Nuclei, dark blue. Basal bodies, purple.

EOSIN - METHYLENE BLUE - BASIC FUCHSIN

A general stain for bacteria, blood and spirochaetæ

Solution required:

Eosin 2% in absolute alcohol ..	2·5 ml.
Methylene blue 3% alcoholic ..	7·5 ml.
Basic fuchsin 10% alcoholic ..	3·0 ml.
Absolute alcohol	12·0 ml.
Distilled water	25·0 ml.

Mix well. Allow to stand two or three days in a closed vessel; then filter.

Technique:

1. Blood and spirochaete smears are allowed to dry in the air before fixation in methyl alcohol. Bacterial smears are fixed by heat in the usual manner.

2. Stain smears for one minute in the above solution.

3. Pour off excess stain and wash thoroughly in distilled water.

4. Drain; blot carefully but thoroughly and examine.

Results:

Bacteria—B. *typhosus* and paratyphoid organisms, pink. *B. coli* and *B. dysenteriae*, lilac. *B. diphtheriae*, pink with lilac granules and bars. *B. influenza* and *Leptothrix* unstained but showing lilac bars and granules. *Meningicocci*, violet to pink.

Blood: Erythrocytes, pink to orange. Neutrophiles, cytoplasm, pink. Lymphocyte cytoplasm, purple; nuclei, blue. Basophiles, purple to dark blue; nuclei, dark purple to black. Eosinophile granules, pink to opaque white. Polychromatophilic cells, red.

Spirochaetes: pink to violet.

FIELD'S STAIN

For thick blood films for malaria parasites

Solutions required:

A. Field's stain, A

B. Field's stain, B

Technique:

1. A thick film is made by spreading two or three drops of blood into a circle of about 2 cm. diameter on a slide.

2. Allow to dry in the air until the films are no longer obviously moist.

3. Immerse the film for one second in Field's stain A; then immediately transfer to a dish of distilled water and wave the slide gently in the water until no more stain comes away and the slide is free of excess stain.

4. Immerse the film for one second in Field's stain B; then rinse by waving gently for two or three seconds in a dish of fresh distilled water.

5. The films should be placed in a vertical position and left to drain and dry in air.

Notes:

Freshly prepared films give the best results.
Staining times may be varied from one to five seconds.

Results:

General ground: creamy yellow, sometimes uniform, sometimes mottled with pale blue.

Leucocytes:—Nuclei, deep blue, sharply defined. Cytoplasm pale blue, vaguely defined. Granules, eosinophilic, large, dull red, well defined. Neutrophilic, small, pale purple, vague.

Malaria parasites: Cytoplasm, blue. Chromatin, dark, purplish red. Pigment, unstained yellow of varying shades depending on the depth of the cytoplasm in which it lies.

FLAGELLA STAIN (Cesares-Gil)

For colon and typhoid organisms

Solutions required:

A. Flagella stain (Cesares-Gil).

B. Carbol fuchsin (Ziehl Neelsen).

Technique:

1. A freshly prepared mixture consisting of 1 vol. of the stain and 1 vol. distilled water is filtered directly onto unfixed films of a young culture (eighteen to twenty-four hours old) and allowed to act for one minute.

2. Wash with distilled water.

3. Stain for five minutes with carbol fuchsin (Ziehl Neelsen), freshly filtered.

4. Wash with distilled water; blot and dry in air.

Results:

Bacteria and flagella, red. Background, very fine granular red precipitate.

FONTANA STAIN

For spirochaetæ

Solutions required:

A.	Acetic acid 1%	100 ml.
	Formalin	10 ml.
B.	Tannic acid	5 gm.
	Phenol 1%	100 ml.
C.	Silver nitrate	5 gm.
	Distilled water	100 ml.

Dissolve; then set aside a few millilitres. The bulk is treated as follows:

Add ammonia drop by drop, with constant shaking, until the brownish precipitate, which is formed, just

re-dissolves. A few drops of the reserved silver nitrate are then added, with shaking, until the final solution is slightly opalescent.

Technique:

1. Air-dried films are flooded with Solution A for one minute; then washed with distilled water.

2. Flood with Solution B and heat until steam arises; then pour off and allow the slide to cool somewhat before rinsing with distilled water.

3. Flood with Solution C; heat till steam rises; then allow the solution to act for one half to one minute.

4. Wash with distilled water; dry and blot in air; and mount before examining as the stain may fade immediately in contact with immersion oil.

Result:

Spirochaetes are stained dark brown to black, against a maroon background.

GENTIAN VIOLET (Noland)

A combined fixative and stain for Protozoa

Solutions required:

Gentian violet 2% aqueous ..	1 ml.
Phenol, saturated aqueous	80 ml.
Formaldehyde 40%	20 ml.
Glycerin	4 ml.

Technique:

1. Place a drop of the culture to be examined on a scrupulously clean slide.

2. Add a drop of the staining solution and mix and spread with a platinum wire loop into a film.

3. Allow to dry; then examine under the oil immersion objective.

Results:

Flagella are stained violet, as are undulating membranes and cirri of Hyportichs.

Note: The technique has been found useful for demonstrating the various stages of Dimastogamoeba from amoeboid to flagellating forms after some of a culture has been kept in rain water for one to several hours.

Reference: Noland, L. E. (1928), *Science*, **67**, 535.

GIEMSA STAIN

For blood, malaria parasites, trypanosomes, etc.

Note: Best results are obtained by buffering the distilled water to 7·2.

Rapid method for films:

1. Fix air-dried films for three minutes in pure methyl alcohol.

2. Stain for five minutes in a mixture consisting of one part of Giemsa stain and two parts of distilled water.

3. Wash with distilled water for one half to one minute; then blot and dry in air.

Rapid method for spirochaetes:

1. Air-dried films are fixed by heat by drawing through the flame.

2. Allow the slide to cool; then flood the slide with a freshly prepared mixture consisting of 10 drops of Giemsa stain to 10 ml. distilled water.

3. Heat the slide gently till steam rises; allow to cool for about twenty seconds; then pour off the stain and repeat the process five or six times.

4. Wash with distilled water; blot dry and mount.

Slow method for films, for demonstrating spirochaetes, trypanosomes, etc.:

1. Air-dried films are fixed for three minutes in pure methyl alcohol.

2. A fresh mixture is prepared by diluting Giemsa stain in the proportion of 10 drops of stain to 10 ml. distilled water. The slide is then placed in a staining jar and left to stain in the diluted Giemsa for sixteen to twenty-four hours; if a staining jar is not available, place a piece of thin glass rod in a Petri dish; lay the slide with one end resting on the rod, film face downwards, in the Petri dish, and pour in sufficient diluted stain to cover the film; then line the Petri dish lid with two sheets of moist filter paper to prevent evaporation, and cover the preparation.

3. Wash with distilled water; blot and dry.

Method for bacterial smears, throat exudate, etc.:

1. Thin, air-dried, unfixed smears are covered with undiluted Giemsa stain for thirty seconds; then a quantity of distilled water, equivalent to five to ten times the volume of stain used, is added and mixed with the stain by gently rocking the slide.

2. Allow the diluted stain to act for two to five minutes; then wash with distilled water; blot and dry.

Thin blood film method:

1. Air-dried films are fixed for five minutes in pure methyl alcohol; then blotted and dried in air.

2. Stain for fifteen to forty-five minutes in a mixture consisting of one part of Giemsa stain and twenty-five parts of distilled water.

3. Wash in distilled water; blot and dry in air.

Results:

Nuclei of the leucocytes are stained reddish purple, while the rest of the leucocytes appear similar to Leishman-stained preparations. Cytoplasm of plasmodia, blue. Chromatin, red.

Thick film method (for demonstrating malaria parasites)*:*

1. A film is prepared by spreading 3 to 5 drops of blood in a circle about 15 mm. diameter over a slide; then without fixation it is allowed to dry on a level surface for eighteen to twenty-four hours at room temperature protected from dust; or for two to three hours in an incubator at 37° C.

2. Stain for forty minutes in a mixture consisting of one part of Giemsa stain and fifty parts of distilled water.

3. Wash for five to ten minutes in distilled water; blot and dry in air.

Results:

Malaria parasites: chromatin, clear red; cytoplasm, clear blue.

Treponema pallida.

Solutions required:

A. Giemsa stain.

B. Sodium carbonate, A.R. 1% in distilled water.

Technique:

1. A lesion is rubbed roughly until it bleeds, with a swab which has been damped with absolute alcohol.

2. While bleeding is taking place, swab the chancre at intervals. After a few minutes a clear fluid exudate will appear from the abraded surfaces.

3. A drop of the clear fluid exudate (free from blood) is taken from the periphery of the lesion with a platinum wire loop, transferred to a scrupulously clean slide, and spread into a film.

4. Allow to dry in the air; then fix in absolute alcohol for fifteen minutes.

5. Pour off any excess alcohol, and allow the last traces to evaporate away in the air.

6. Stain for fifteen to thirty minutes in a mixture consisting of 10 ml. distilled water buffered to 7·2, and 0·5 ml. Giemsa stain.

7. Rinse in running tap water for a few seconds holding the slide with the film facing downwards.

8. Blot dry, and examine.

Results:

The spirochaetes are stained reddish violet. Nuclei of the leucocytes reddish purple, while the rest of the leucocytes and pus cells appear similar to Leishman-stained preparation. Cytoplasm of plasmodia, blue; chromatin, red. Erythrocytes, yellowish pink.

GIEMSA STAIN - MAY-GRUNWALD STAIN

For blood and parasites

Solutions required:

A. May-Grunwald stain.

B. Giemsa stain.

Technique:

1. Unfixed air-dried films are stained for three minutes in May-Grunwald stain; then an equal volume of distilled water is added and mixed with the stain by rocking the slide. The diluted stain is then allowed to act for one minute; then drained off, without washing.

2. Stain for ten to fifteen minutes in a mixture consisting of 10 drops of Giemsa stain in 10 ml. distilled water.

3. Differentiate for about five seconds, with distilled water, examining under the microscope to ensure that differentiation is complete.

4. Blot and dry in air; mount.

Results:

As Giemsa stain but with more intense colouring.

GRAM'S STAIN

For bacterial smears

Solutions required:

A. Aniline gentian violet or carbol gentian violet.

B. Gram's iodine.

C. Carbol fuchsin (Ziehl Neelsen) .. 1 volume
Distilled water .. .. .. 9 volumes

Technique:

1. Air-dried bacterial films are fixed by passing through the bunsen flame in the usual manner.

2. Stain with aniline gentian violet or carbol gentian violet for two to three minutes.

3. Pour off stain and flood the film with Gram's iodine for one minute.

4. Blot and dry in air.

5. Decolorize with absolute alcohol (if aniline gentian violet has been used) until no more colour comes out; if carbol gentian violet has been used, decolorize with aniline xylol instead of alcohol.

6. Wash with water; drain.

7. Counterstain with a mixture consisting of 1 volume of Ziehl Neelsen's carbol fuchsin and 9 volumes distilled water.

8. Wash with distilled water; blot and dry in air.

Results:

Gram-positive organisms, violet. Gram-negative organisms, red with pink nuclei.

GRAM'S STAIN

Jensen's modification

Solutions required:

A. Methyl violet 6B (Jensen).

B. Lugol's iodine.

C. Neutral red (Jensen).

Technique:

1. Air-dried bacterial films are fixed as above.

2. Stain for twenty to thirty seconds with 0·5% methyl violet 6B in distilled water.

3. Pour off; wash film with Lugol's iodine; and allow the iodine to act on the film for one half to one minute; then pour off.

4. Wash with absolute alcohol until no more colour comes out of the film; then wash with distilled water and drain.

5. Counterstain with Jensen's neutral red (0·1 gm. neutral red, 0·2 ml. 1% acetic acid, 100 ml. distilled water) for thirty to sixty seconds.

6. Wash with distilled water; drain, blot and dry in air.

Note: Ziehl Neelsen's carbol fuchsin diluted 1 : 15 with distilled water may be used (staining time, twenty to thirty seconds) for routine work. For demonstrating gonococcus and other intracellular Gram-negative bacteria, however, neutral red should be used.

GRAM'S STAIN

(Kopeloff and Berman modification)

For bacterial smears

Solutions required:

A. Crystal violet 1% aqueous.

B. Sodium bicarbonate 5% aqueous.

C.

Potass. iodide	2 gm.
Iodine	1 gm.
Distilled water	89 ml.
N/1 NaOH	10 ml.

D. Equal volumes of acetone and ether.

E. Safranin 2% aqueous.

Technique:

1. Thin, unfixed, air-dried smears are stained for five to ten minutes in a mixture consisting of 3 ml. Solution A, and 0·8 ml. of Solution B.

2. Rinse with distilled water then stain with Solution C for two to five minutes.

3. Wash with tap water and drain and blot water from the surface of the film, but do not allow the films to dry.

4. Decolorize by adding Solution D drop by drop for about ten seconds, until hardly any more colour comes out: this takes about ten seconds.

5. Dry in air and counterstain in Solution E for five to ten seconds.

6. Wash in tap water, dry and examine.

Results:

Gram-positive organisms, blue. Gram-negative, red.

GRAM'S IODINE - ANILINE GENTIAN VIOLET

For spirochaetae of Vincent's Angina and for *Trep. pallidum*

Solutions required:

A. N/20 HCl.

B. Gram's iodine.

C. Aniline gentian violet.

Technique:

1. Spread scrapings from ulcer on a slide; then allow to dry in the air before fixing by heat in the usual manner.

2. Immerse in N/20 HCl for 10 seconds.

3. Wash in running water for 5 seconds.

4. Immerse in Gram's iodine for 5–10 seconds.

5. Wash with water.

6. Stain with aniline gentian violet for 5–10 seconds.

7. Wash with water.

8. Drain and blot dry.

9. Mount if desired, in D.P.X., Clearmount, or Emexel and examine under the oil immersion objective.

Results:

Spirochaetes are stained deep violet.

eference: Bailey, H. D. (1937–38), *J. Lab. and Clin. Med.*, **23**, 960.

HAEMATOXYLIN (Phosphotungstic, Mallory) For Entamoeba

Solutions required:

A. Absolute alcohol .. .. 1 volume
Mercuric chloride, saturated, aqueous .. .. .. 2 volumes

B. Iodine 1% alcoholic.

C. Phosphotungstic acid haematoxylin (Mallory).

Technique:

1. Thin smears are made on scrupulously clean slides and fixed while still moist in the mercuric chloride solution for fifteen minutes.

2. Wash and immerse in water for a few seconds.

3. Flood slides with the iodine solution and leave thereon for three minutes.

4. Rinse thoroughly with distilled water until the iodine colour is completely extracted.

5. Stain for half an hour in phosphotungstic acid haematoxylin.

6. Rinse in water.

7. Dehydrate with 95% and absolute alcohol; clear in xylol and mount.

Results

Nuclei, deep blue; cytoplasm, pale blue.

HAEMATOXYLIN (Weigert)—BORDEAUX RED

For permanent preparations of anopheline midgut

Solutions required:

A. Absolute alcohol .. .. 45 ml.
Formalin .. .. .. 3·5 ml.
Glacial acetic acid .. .. 1·5 ml.

B. Weigert's haematoxylin, A.

C. Weigert's haematoxylin, B.

D. Bordeaux Red 0·05% in absolute alcohol.

Technique:

1. The specimen together with dissecting medium is placed on a slide and covered with a coverglass; it is then fixed for seven minutes by drawing Solution A under the coverglass on one side, whilst the dissecting medium is drawn off on the opposite side with a piece of filter paper.

2. Flood the slide while the coverglass is still on with Solution A for five minutes.

3. Very carefully lift the coverglass with a needle so that the gut adheres either to the slide or to the coverglass.

4. Carefully wash the specimen with 70% alcohol; then cover it with the alcohol for five minutes.

5. Cover with 50% alcohol for five minutes.

6. Stain for thirty seconds with a freshly prepared mixture consisting of 0·5 ml. each Solutions of B and C, and 25 ml. 50% alcohol.

7. Very carefully wash with 50% alcohol; cover with 70% for five minutes, followed by 95% for five minutes.

8. Stain for ten seconds in Solution D.

9. Flush with absolute alcohol, very carefully; clear in xylol and mount.

HAEMATOXYLIN EOSIN - INDIGO CARMINE

For the differential staining of vaginal smears

Solutions required:

A. Haematoxylin (Harris).

B. Absolute alcohol .. .. 70 ml.
Distilled water .. .. .. 28·8 ml.
Strong ammonia solution .. 1·2 ml.

C. Indigocarmine .. .. .. 0·25 gm.
Eosin, yellowish .. .. .. 0·75 gm.
Distilled water .. .. .. 1 ml.
Chloroform .. .. .. 5 drops

Technique:

1. Make smears on scrupulously clean slides; allow to dry.
2. Immerse in 95% alcohol for three minutes.
3. Transfer to and immerse in distilled water for one minute.
4. Transfer to and immerse in haematoxylin (Harris) for twenty minutes.
5. Wash with running tap water for two minutes.
6. Immerse in Solution B for a few seconds.
7. Stain in Solution C over-night.
8. Rinse quickly in tap water.
9. Dehydrate with 70%, 90% and absolute alcohol.
10. Clear in xylol.
11. Mount in Cristalite.

Results:

Nuclei of epithelial cells are stained grey, while the cytoplasm is violet. Cornified cells, brilliant pink. Erythrocytes, pink.

IODINE - EOSIN

For intestinal amoebae and flagellates in faeces

Solutions required:

A. Saturated solution of iodine in 5% aqueous potassium iodide.
B. Eosin, yellowish, aqueous 10%.
C. Equal volumes of Solutions A and B.

Technique:

1. Spread a loopful of faeces on a slide with one or two drops of Solution C.

2. Mix well and cover with a coverslip before examining under the microscope.

Results:

Cysts of amoebae and flagellates are stained yellow to greenish yellow against a red background, while glycogen bodies within the cysts are stained brown.

JANUS GREEN B

For staining oocysts of Coccidia

Solutions required:

A. Sodium chloride, saturated, aqueous.

B. Janus green B aqueous 0·01%.

C. Eosin, yellowish 10% in tap water.

Technique:

1. The faecal material is strained through a double layer of cheese muslin.

2. Centrifuge in ordinary pointed tubes.

3. Add more water and repeat the process twice, to remove most of the debris.

4. Add sodium chloride solution, shake well and centrifuge, when the oocysts will appear on the surface.

5. Collect oocysts and transfer to a slide.

6. Cover with a coverslip.

7. Replace the sodium chloride with glacial acetic acid by drawing the acid under the coverslip by means of filter paper.

8. Gently warm the slide for five or ten minutes, taking care not to evaporate the acid.

9. Replace the acid with a freshly prepared solution of janus green B (Solution B above) by drawing the stain under the coverslip by means of a piece of filter paper; then allow the stain to act for ten minutes.

10. Wash and stain with the eosin solution for five minutes.

11. Wash in water.

12. Blot off excess water and seal with petroleum jelly or with glycerine jelly.

Note: The whole of the process of staining and washing can be observed under the low-power objective; but studying is recommended under the oil-immersion objective.

Results:

Oocyst jelly is stained red while the walls may appear reddish, and the structures within the sporozites are rendered visible.

Abstract *Science*, 73, 212–13, 1931, H. B. Crough and E. R. Becker.

JANUS GREEN, B - NEUTRAL RED

For supravital staining of blood

Solutions required:

A. Janus green, B vital stain 0·4% in neutral absolute alcohol.

B. Neutral red chloride, vital stain 0·25% in neutral absolute alcohol.

C. Solution A .. .. 0·07 ml.
Solution B .. .. 1·75 ml.
Absolute alcohol, neutral .. .. 10 ml.

Note: Solutions A and B are stable, but solution C deteriorates after a few hours, and it should, therefore, be prepared as and when required, for immediate use.

Technique:

1. Scrupulously clean, dry slides are flooded with solution C.
2. Drain and leave to dry.
3. Place a small drop of blood on each slide.
4. Cover the blood with scrupulously clean, dry coverslips and allow the blood to spread.
5. Seal the edges of the coverslips with soft paraffin wax (M.P. 38° C.), and examine under the microscope.

Results:

Basophilic granules: brilliant scarlet. Eosinophilic granules:

yellow to light orange. Neutrophilic granules: salmon colour. Mitochondria: small blue dots or rods. Nuclei: unstained.

Note: The proportion of the two stains may be varied to suit the particular specimen; for instance, specimens very rich in cells, such as leucaemic blood, need more concentrated mixtures of the stains.

JENNER STAIN

For cytological examination of blood

Technique:

1. Air-dried, unfixed blood films are stained for three minutes, face downwards to prevent precipitate depositing on the film and obscuring the picture.

2. Wash with distilled water until the film appears pink to the naked eye.

3. Blot dry and examine.

Results:

Neutrophile granules, pink. Oxyphile granules, brownish red. Basophile granules, purple. Nucleoli (plasmosomes), pink. Cytoplasm of partially haemoglobinated precursors of erythrocytes, varying shades of purple. Mature erythrocytes, deep pinkish orange.

LEISHMAN STAIN

For blood, malaria parasites, trypanosomes, etc.

This stain offers a simple and precise method of staining blood for diagnostic purposes.

Best results are obtained by buffering the distilled water to pH 6·6–7·0.

Technique for blood films:

Fixation is unnecessary unless the films are to be kept in stock for any length of time, in which case they should be fixed for five minutes in pure methyl alcohol at room temperature.

Thin film method:

1. Air-dried films are stained, without fixing, for one minute with five to ten drops of the stain; then double the quantity of distilled water (i.e. ten to twenty drops) is added and mixed by rocking the slide gently.

2. Allow this diluted stain to act for five to ten minutes; then pour off.

3. Wash gently with distilled water; then differentiate by flooding the slide with distilled water and allowing the water to remain on the slide for about one half to one minute, until the film appears pink to the naked eye.

4. Pour off; blot gently and dry in air.

Results:

Similar to Wright's stain.

Thick film method:

This method should be employed in searching for blood parasites when negative results have been obtained by the thin film method.

1. A film is prepared by spreading three to five drops of blood in a circle about 15 mm. diameter over a slide; then without fixation, it is allowed to dry at room temperature from twelve to twenty-four hours, protected from dust. The time required for drying may be very considerably shortened by placing the slide in an incubator at 37° C.

2. Remove the haemoglobin by placing the film face downwards in a dish of distilled water at room temperature.

3. Fix in acid alcohol for five to fifteen minutes.

4. Wash well with distilled water; blot gently and dry in air.

5. Stain in accordance with the method described above for thin films.

Bacterial smears, throat exudate, etc.:

1. Unfixed, air-dried smears are stained with 1 ml. of the stain for two minutes; then without pouring off:

2. Add an equal volume of distilled water, rock the slide to mix; then allow the diluted stain to act for one minute.

3. Pour off; blot and dry in air.

Results:

Bacteria are stained dark blue; capsules, light blue. Mucus, red.

LUGOL'S IODINE - EOSIN

For differentiating between spores and vegetative forms in bacterial smears

Solutions required:

A.	Lugol's iodine	40 ml.
	Eosin yellowish	2 gm.
	Phenol cryst.	2 gm.
B.	Methylene blue 1% aqueous ..	10 ml.
	Absolute alcohol	5 ml.
	Distilled water	85 ml.

Dissolve by shaking thoroughly in a conical flask; filter before use.

Technique:

1. Fix smears by flaming in the usual manner.

2. Place slides, smear upwards, on the corner of a tripod; flood with the iodine-eosin stain (Solution A) and heat the underside of the slide for five minutes, with a small flame, until the liquid begins to boil.

3. Remove the flame momentarily, applying it again the instant the liquid ceases to boil; repeat this process two or three times.

4. Pour off excess stain; allow the slide to cool somewhat; then plunge quickly into distilled water.

5. Stain for two minutes in the methylene blue solution.

6. Rinse with distilled water.

7. Drain; blot dry; examine.

Results:

Spores, deep pink. Vegetative form, blue.

MACCHIAVELLO'S STAIN

For Rickettsiae

Solutions required:

A. Basic fuchsin .. .. .. 0.25 gm.
Distilled water adjusted to pH 7·2–7·4 with alkali .. .. .. 100 ml.
This solution should be freshly prepared and filtered.

B. Citric acid 0·5% aqueous.

C. Methylene blue 1% aqueous.

Technique:

1. Spread smears of the infected tissue on scrupulously clean slides.

2. Fix by flaming gently.

3. Stain for four minutes with solution A.

4. Pour off the excess stain; then wash to preparation rapidly with the citric acid solution.

5. Wash with tap water.

6. Stain for a few seconds with the methylene blue.

7. Wash; dry; examine.

Results:

Rickettsiae, red. Tissue cells, blue.

MALACHITE GREEN

A simple method of staining spores

Solutions required:

A. Malachite Green 5% aqueous.

B. Safranin 0·5% aqueous.

Technique:

1. Bacterial films are dried by heating gently over a bunsen flame.

2. Heat on a steam bath till water condenses in droplets on the bottom of the slides.

3. Flood films with Solution A and heat for one minute; then pour off excess stain and wash well with water.

4. Counterstain thirty seconds in Solution B; then wash with water; blot dry and examine.

Results:

Spores, green. Vegetative forms, red.

MALACHITE GREEN - BASIC FUCHSIN

For staining yeasts or bacteria

Solutions required:

A. Basic fuchsin 10% alcoholic .. 0·5 ml.
Malachite green 0·5% aqueous .. 100 ml.

Technique:

1. Spread smears on slides with a platinum wire loop in the usual manner.

2. Place slides, smear facing upwards, on the corner of a tripod.

3. Flood slide with the staining solution.

4. Heat the underside of the slide very gently over a small bunsen flame some distance away from the slide until steam rises.

5. Pour off excess stain after a minute or so.

6. Wash well with distilled water.

7. Drain; blot dry, and examine.

Results:

Spores are stained greenish blue, while vegetative forms appear violet to pink.

Reference: Gray, P. H. H. (1941), *Nature*, 147, 329.

MALACHITE GREEN - PYRONIN - CRYSTAL VIOLET (Sandiford)

A contrast stain for gonococci and meningococci being a Gram stain with modified Pappenheim as counterstain

Solutions required:

A. Crystal violet 1% in 98% alcohol 20 ml.
Ammonium oxalate 1% aqueous 30 ml.

B. Lugol's iodine.

C. Malachite green 1% aqueous .. 5 ml.
Pyronin, B 1% aqueous .. .. 15 ml.
Distilled water .. .. .. 80 ml.

Technique:

1. Stain for thirty seconds with Solution A.

2. Pour off and wash for thirty seconds with Solution B; then pour off and blot.

3. Decolorize for three or four seconds with acetone; then wash with water.

4. Stain for two minutes with Solution C.

5. Flood with water, but do not wash; blot dry and examine.

Results:

Cells and nuclei, bluish green. Gram-positive organisms, purplish black and *Neisserieae* red.

MAY-GRUNWALD STAIN

This stain may be used alone for the cytological examination of blood films, but it is not suitable for parasites. The technique is the same as that for Jenner stain. Its chief use is in conjunction with Giemsa stain (*see* page 368).

METHYL GREEN - PYRONIN (UNNA-PAPPENHEIM)

For gonococci

Solutions required:

A. Methyl green .. .. .. 1 gm.
Pyronin B .. .. 0·2 gm.
Phenol crystals .. .. .. 2 gm.
Absolute alcohol .. .. .. 10 ml.

Glycerine .. .. 20 ml.
Grind together in a mortar and pestle adding:
Distilled water .. .. .. 100 ml.
in portions of about 10 ml. over a period of about 20 minutes until solution is complete; then filter.

Technique:

1. Spread smears on scrupulously clean slides and fix by flaming in the usual way.
2. Flood slides with the stain and heat till steam rises, then remove flame and allow the warm stain to act for about 45 seconds.
3. Pour off excess stain.
4. Wash with distilled water; then immerse in distilled water for about 30 to 60 seconds.
5. Shake off excess distilled water; then blot dry and examine.

Results:

Gonococci are stained deep red while other bacteria, except those of the Neisseria group, are faint purple or almost unstained. Cytoplasm of pus cells pink while the nuclei are green.

METHYL VIOLET 10B

For the direct staining of elementary bodies

Solutions required:

A. Methyl violet 10B aqueous 1%.

B. Sodium bicarbonate.. .. .. 2 gm.
Distilled water (cold) .. .. 100 ml.
Dissolve by shaking.

Technique:

1. Scrupulously clean slides are prepared by rinsing in nitric acid, followed by water; then alcohol-ether.

2. Spread films, of a more or less pure suspension, on slides. If much protein is present rinse the films with saline and then with distilled water before allowing to dry in the air or in the incubator.

3. Fix by flaming in the usual way.

4. Mix equal volumes of solutions A and B and filter at once onto the slides .

5. Heat the slides over the bunsen flame or on a hot plate until steam rises, but do not allow the stain to boil.

6. Allow the hot stain to act for three to five minutes; then pour off excess and allow the slide to cool somewhat before rinsing with distilled water.

7. Dry thoroughly; then mount in cedarwood immersion oil or in liquid paraffin.

Results:

Elementary bodies are stained sharply a light violet colour.

Note: Instead of methyl violet the following solution may be used, in which case the elementary bodies will stain a pinkish colour: Basic fuchsin 10% in absolute alcohol 1 volume; distilled water 9 volumes.

Reference: Gutstein, M. (1937); *J. of Path. and Bact.*, **45**, no. 1, 313–4.

METHYLENE BLUE - CARBOL FUCHSIN

For flagella and capsules in bacterial films

Solutions required:

A. Potash alum, saturated aqueous 40 ml.
Tannic acid aqueous 10% .. 40 ml.
Methylene blue, saturated in absolute alcohol .. .. .. 10 ml.

Note: This solution deteriorates after a week or ten days.

B. Carbol fuchsin (Ziehl Neelsen) .. 5 ml.
Distilled water .. .. .. 45 ml.

Technique:

1. Smears are made on scrupulously clean slides by spreading a minute speck of the culture with distilled water.

2. Allow to dry and fix by flaming in the usual manner.

3. Stain with the methylene blue solution (formula as above) for ten minutes at about 40° C.

4. Pour off excess stain and rinse the preparation in distilled water.

5. Counterstain for about five minutes with the diluted carbol fuchsin solution.

6. Pour off excess; rinse in distilled water; blot dry and examine.

Results:

Flagella are stained blue, while capsules are red.

METHYLENE BLUE - GENTIAN VIOLET

For gonococci

Solution required:

Methylene blue	0·5 gm.
Gentian violet	0·5 gm.
Distilled water	100 ml.

Technique:

1. Thin smears of the secretion to be examined are fixed by flaming.

2. Stain for thirty seconds with the above solution; then wash and dry.

Results:

Gonococci, dark violet. Nuclei of leucocytes, dark blue. Cytoplasm of leucocytes, pale reddish violet. Nuclei of epithelial cells, deep blue.

METHYLENE BLUE - SAFRANIN

For polar bodies of bacteria

Solutions required:

A. Methylene blue (Neisser).

B. Safranin 1% aqueous.

Technique:

1. Air-dried smears are fixed by flaming in the usual manner.

2. Immerse in the methylene blue solution for five minutes.

3. Pour off excess stain and carefully wash the preparation in running water.

4. Drain off excess water; then cover the preparation with safranin solution and allow the stain to act for one to two minutes.

5. Rinse well with distilled water; drain; blot dry; examine.

Results:

The polar bodies appear deep blue, while the bacterial cells are distinctly red.

NEW FUCHSIN - CONGO RED

For bacterial cell walls, particularly *E. Coli* and *B. Cereus*

Solutions required:

A. New fuchsin 1% aqueous.

B. Congo Red .. .. 1 gm.
Buffer solution pH 9·5. .. .. 100 ml.
Warm to dissolve

Technique:

1. Smears of the organisms are made on slides in the usual manner, with a wire loop, 1 mm. diameter.

2. Allow the smears to dry in the air without the use of heat.

3. Stain in the new fuchsin solution for three to four minutes.

4. Pour off excess stain: then rinse the slides gently until the colour ceases to come out.

5. Shake off excess water and set the slide aside to dry in the air.

6. Place the air dried slides on a warm plate at 50° C. for ten to twenty seconds.

7. Remove from the hot plate and uslng a wire loop 1 mm. in diameter, add a loopful of the congo red solution and spread this

stain, by means of the wire loop, over the smear. Allow the stain to act for about one half to one minute.

8. Wash off excess stain with distilled water.

9. Shake off excess distilled water; then allow the smear to dry thoroughly in the air before examining under the oil immersion objective.

Results:

Cell walls stained red.

Note: Walls in the early stages of formation are not stained. Cross walls are stained in the older portion of a filament of *B. cereus* but not in the younger portions. The end cell of a filament has relatively heavy side walls but the wall becomes much thinner around the free end of the cell. The stain does not show cross walls in chains of *E. Coli*.

A further differentiation of the stained material may be made by exposing the stained specimen to the fumes of hydrochloric acid which will change the colour of the free congo red to blue.

Reference: Chance, H. L. (1953), *Stain Tech.*, **28**, 205–7.

NEWMAN'S STAIN

A single solution for defatting and staining milk smears for the enumeration of milk bacteria

Solution required:

Newman's Stain	
Methylene blue	1 gm.
Absolute alcohol	54 ml.
Tetrachlorethane	40 ml.
Acetic acid, glacial	6 ml.

Place the methylene blue with the alcohol in a flask; then plug the neck of the flask lightly with cotton-wool. Heat water till it boils in a water bath; then turn off the flame and place the flask in the hot water until the alcohol just begins to boil. Allow the solution to cool after swirling the liquid round inside the flask until all the methylene blue has gone into solution. Filter into the stock bottle; add the trichlorethane; replace the stopper or cork and shake well.

Technique:

1. Spread 0·01 ml. of the milk over a carefully measured square centimetre on a scrupulously clean slide.

Note: The area can best be measured out by marking it on a piece of white paper and placing the slide over the square.

2. Allow the film to dry; then fix in pure methyl alcohol for one minute.

3. Apply the stain and allow it to act for about thirty seconds.

4. Wash with water.

5. Dry and examine, using a micrometre eyepiece and an oil immersion lens.

Results:

Bacteria, deep blue. Background, pale blue.

NILE BLUE SULPHATE

For protozoa and yeasts

Solution required:

Nile blue sulphate 0·1% aqueous.

Technique:

1. Stain from five to ten minutes according to the material and depth of staining desired.

2. Rinse in distilled water; dry, and examine.

Note: This stain may also be employed for living amphibian eggs and for Hydra.

PAPANICOLAOU STAIN

For improved differentiation of the cells of vaginal smears

Staining solutions:

A. Ehrlich or Harris haematoxylin.

B. Orange G .. .. .. 0·2 gm.
Alcohol 95% .. .. .. 100 ml.
Phosphotungstic acid .. .. 0·015 gm.

C. Papanicolaou stain, powder .. 0·7 gm.
Alcohol 95% .. .. .. 100 ml.

Place in a flask, plug the neck lightly with cotton-wool; then heat, on a waterbath until dissolved. Cool, then filter.

Technique:

1. Wet smears are fixed in a mixture of equal volumes of ether and absolute alcohol for five to fifteen minutes.

2. Rinse successively in 90%, 70% and 50% alcohols and distilled water.

3. Stain in Ehrlich's haematoxylin for five to ten minutes.

4. Rinse in distilled water; differentiate in 0·5% hydrochloric acid.

5. Rinse in distilled water; then leave for one minute in dilute lithium carbonate solution (three drops saturated lithium carbonate to 100 ml. distilled water).

6. Rinse thoroughly in distilled water and wash successively in 50%, 70%, 95% alcohol.

7. Stain for one minute in Orange G solution (prepared as above).

8. Rinse thoroughly in 95% alcohol to remove all excess stain.

9. Stain for two minutes in Papanicolaou stain; then rinse five to ten times in each of three jars of 95% alcohol.

10. Rinse in absolute alcohol; clear in xylol and mount.

Results:

Nuclei, red. Erythrocytes, orange. Cornified cells, red, pink or orange. Basophile cells, blue or green.

PEROXIDASE REACTION (Cowdry's method)

For blood films

Solutions required:

A. Copper sulphate crystals .. 0·5 gm.
Distilled water .. .. .. 100 ml.

B. Benzidine base, pure .. .. 0·2 gm.
Distilled water .. .. .. 200 ml.
Filter and add 4 drops hydrogen peroxide (20 vols.) to the filtrate; store in a dark bottle.

C. Safranin .. .. 1% aqueous

Technique:

1. Air-dried blood films are flooded with Solution A for one minute. Pour off; then without washing and while still wet:

2. Flood with Solution B and leave for two minutes; then rinse in tap water.

3. Counterstain in Solution C for one minute.

4. Wash with tap water; blot and dry.

Results:

Peroxidase granules, blue. Nuclei of leucocytes, orange-red.

Note: Unmounted specimens keep for many months without fading.

PICRO METHYL BLUE - EOSIN

For urinary casts

Abstract *J. of Lab. & Clin. Med.* (U.S.A.), 22, 853, 1937, Jeanette Allen Behre, and William Muhlberg.

Solutions required:

A. Eosin yellowish 0·5% aqueous.

B. Methyl blue 1% aqueous .. 1 ml.
Picric acid saturated, aqueous .. 10 ml.
Glycerine .. .. 10 drops

Technique:

1. The urine is centrifuged and the supernatant liquid decanted as usual for microscopic examination.

2. One drop of eosin solution is added to the sediment and mixed by shaking from side to side for one to two minutes.

3. Two drops of Solution B are added and mixed. The colour of the sediment should now be distinctly blue-green; if it is reddish brown more of Solution B should be added till the blue-green colour is obtained, but too much should be avoided.

4. Some of the stained sediment is then transferred to a slide, covered with a coverglass and examined.

Note: The amounts of the two stains may be varied according to the amount and particular character of the sediment present. More eosin may be added if the cells have not been stained sufficiently red. Enough of the methyl blue should be added to stain the casts a distinct blue, but too much will stain them too dark.

More permanent slides may be made by adding more glycerine to the sediment and sealing the edges of the cover glass with Vaseline or balsam.

This technique brings out the detailed structure of casts and castlike bodies in a remarkable way. It does not furnish a differential stain since all the mucous material is also stained blue.

Results:

Hyaline casts are stained a clear blue of varying intensity. The more irregularly shaped bodies, sometimes classed as cylindroids, are similarly stained. An irregular distribution of material, a " mealy " structure, or a striated appearance sometimes becomes evident in bodies which appear perfectly homogeneous before staining. Mucous threads and amorphous material are also stained blue; they are clearly differentiated from the cast-like bodies by their structure. Granular material is usually stained darker. Mixed, fine granular casts present a striking picture of fine, dark granules powdered over the light blue, hyaline body. Some coarsely granulated casts are stained deep blue; the granules of the others are yellow, orange or dark reddish brown. Renal epithelial cells are usually red, sometimes orange or yellowish. Red blood cells are stained a brighter red. Pus cells are usually red, occasionally blue. Epithelial cells from the urinary passages are either red or blue.

Fat globules are unstained. In cells which are undergoing fatty degeneration, the fat cells are seen strikingly against reddish

cellular material. The picric acid gives a light yellowish background.

Often the stain reveals the presence of lightly stained mucus-like envelope, apparently covering the cast material. This material is often clearly seen at the end of a cast which has apparently been squarely broken off.

PINACYANOL - NEUTRAL RED

For supra-vital staining of blood

Note: Pinacyanol is superior to janus green in that it does not fade: it is very selective for mitochondria, and it does not inhibit the effect of neutral red. It has the disadvantage, however, of being extremely costly.

Solutions required:

Stock solutions:

A. Pinacyanol 0·1% in absolute alcohol.

B. Neutral Red 0·1% in absolute alcohol.

Staining solutions:

C. *for mitochondria only*

Solution A	1 ml.
Absolute alcohol	20 ml.

D. *for mitochondria, nuclei and other cell granules*

Solution A	1 ml.
Solution B	2 ml.
Absolute alcohol	20 ml.

Notes:

(*a*) Solutions C and D deteriorate after a few hours and should, therefore, be prepared only as and when they are required for immediate use.

(*b*) The proportions and dye concentrations of either of these two solutions may be varied to suit particular specimens.

Technique:

1. Scrupulously clean dry slides are flooded with solution C or D, whichever is required.

2. Drain and leave to dry, then use as follows (within a few hours at latest in the case of solution D) as the dried dye combination does not keep well.

3. Place a drop of blood on each slide.

4. Cover with scrupulously clean coverslip and allow the blood to spread.

5. Seal the edges of the coverslips with soft paraffin wax (M.P. 38° C.), and examine under the microscope.

Results:

With Solution C (Pinacyanol only)

Mitochondria in still living and motile cells are stained deep blue to violet.

With Solution D

Mitochondria stained as above; nuclei and other cell granules are stained red.

Reference: Hetherington, D. C. (1936), *Stain Tech.*, **11**, 153–4.

PONDER'S STAIN (Kinyoun's modification)

For differentiation of metachromatic granules of diphtheria organisms

Solution required:

Toluidine blue	0·1 gm.
Azur A	0·01 gm.
Methylene blue	0·01 gm.
Alcohol 95%	5 ml.
Dissolve; then add:	
Distilled water	120 ml.
Glacial acetic acid	1 ml.

Allow the solution to stand for twenty-four hours before use.

Technique:

1. Air-dried films are fixed by flaming; then stained for two to seven minutes in the above solution.

2. Wash in tap water; then blot dry and examine.

Results:

Granules, dark red. Body of cells, pale blue. Other bacteria, pale.

PYRONIN - ALPHANAPHTHOL (Graham)

For oxidase granules in blood smears

Solutions required:

A. α-Naphthol, pure .. .. 1 gm.
Alcohol 40% .. .. .. 100 ml.
Hydrogen peroxide 20 vols. .. 0·2 ml.

Note: This solution deteriorates in four or five days when the hydrogen peroxide has been added, and it is, therefore, better not to add the hydrogen peroxide until the solution is required for immediate use.

B. Pyronin .. .. 0·1 gm.
Alcohol 40% .. .. .. 96 ml.
Aniline oil.. .. .. .. 4 ml.

C. Methylene blue 0·5% aqueous.

Technique:

1. Freshly spread air-dried blood smears are fixed in 10% formalin for two minutes; then washed well in distilled water.

2. Stain for five minutes in Solution A (as above); then wash for fifteen minutes in running water.

3. Stain for two minutes in Solution B (as above); then wash in distilled water.

4. Stain for one half to one minute in Solution C (as above); then wash with water; blot and dry thoroughly; mount in balsam.

Results:

Neutrophile granules, which give the oxidase reaction, are stained purple to red; while eosinophile granules are lighter red, larger and more refractile. Basophile granules are stained a deep purple; cell nuclei, blue; cytoplasm, pale blue; erythrocytes appear greenish yellow to pink.

SAFRANIN - LIGHT GREEN

For spirochaetes, etc.

Solutions required:

A. Tannic acid .. .. .. 100 gm.
Alcohol 95% .. .. .. 100 ml.

B. Acetic acid, glacial .. .. 7·5 ml.
Formalin .. .. 100 ml.

C. Safranin 1% aqueous.

D. Light green 1% aqueous.

Technique:

1. The material suspected of containing spirochaetes is placed in a drop of 5% acetic acid on a slide. The slide is then inverted over the cavity of a well slide and placed in an incubator for fifteen minutes.

2. Spread the drop with a wire loop and allow it to dry in the air.

3. Flood the film with a freshly prepared mixture consisting of 1 volume Solution A and 2 volumes of Solution B and steam from two to five minutes.

4. Wash with warm distilled water; then stain with Solution C for four to eight minutes.

5. Wash with distilled water; counterstain with light green.

SCHORR'S STAIN

For vaginal smears

Staining solutions:

A. Harris's haematoxylin.

B. Biebrich Scarlet .. .. .. 1 gm.
Orange G .. .. .. 0·4 gm.
Distilled water .. .. .. 99 ml.
Glacial acetic acid .. .. 1 ml.

C. Phosphomolybdic acid .. .. 2·5 gm.
Phosphotungstic acid .. .. 2·5 gm.
Distilled water .. .. .. 100 ml.

D. Fast Green FCF .. .. .. 0·25 gm.
Acetic acid, 0·3% .. .. 100 ml.

Technique:

1. From the fixing solution, carry through alcohols to water; stain in Solution A for two minutes; then wash in running water for five minutes.
2. Stain for one minute in Solution B; then wash in water.
3. Mordant in Solution C for one minute; then wash in water.
4. Stain in Solution D for two minutes; then without washing:
5. Differentiate for one minute in 1% acetic acid.
6. Dehydrate in the usual manner; clear in xylol and mount in dammar-xylol.

Note: Solution A may be omitted in certain cases, as in the routine treatment of menopause with oestrogens. With this omission smears can be stained in five minutes.

Results:

Cornified cells, orange-red. Non-cornified cells, green.

SCHORR'S STAIN
(Single solution)
For vaginal smears

Solutions required :

A. Harris Haematoxylin
B. Schorr's Stain (Single)

Technique:

1. Fix wet smears one to two minutes in equal parts of absolute alcohol and ether: then pass into two changes of absolute alcohol, followed by 80% and 66% alcohol.

2. Rinse with distilled water for ten seconds; then stain in solution A for two minutes.

3. Wash in running water for five minutes; then stain with solution B for two minutes.

4. Dehydrate with 66%, 80% and absolute alcohol; then clear in xylol and mount.

Results:

Cornified cells, orange-red. Non-cornified cells, green.

SUDAN BLACK B - EOSIN - METHYLENE BLUE

For lipoid granules in leucocytes

Solutions required:

A. Sudan Black B, saturated in ethylene glycol.
Note: This should be stored in a well-stoppered bottle.

B. Eosin, yellowish 1% in 70% alcohol.

C. Methylene blue, saturated, aqueous.

Technique:

1. Air-dried blood films are fixed for thirty seconds in pure methyl alcohol.

2. Stain for half an hour in the sudan black solution in a stoppered staining jar.

3. Rinse in water.

4. Rinse thoroughly in 70% alcohol.

5. Counterstain for thirty seconds with the eosin solution.

6. Wash in tap water.

7. Stain in methylene blue solution for three minutes.

8. Rinse with distilled water.

9. Drain and blot thoroughly; examine under the oil-immersion objective.

Results:

Lipoid granules are stained an intense black, whilst nuclei are stained blue, and erythrocytes, red.

SUDAN BLACK - SAFRANIN

For demonstrating fat in bacteria

Solutions required:

A. Sudan black, B saturated in 70% alcohol.

B. Safranin O, aqueous 1%.

Technique:

1. Filter about ten drops of the sudan black solution, which should be two or three days old, into a test-tube.

2. Emulsify bacteria from a slant culture directly into the filtered sudan black in the test-tube.

3. Allow the tube to stand undisturbed for fifteen to twenty minutes until the precipitate has settled.

4. On a scrupulously clean slide place a loopful of the emulsion from the top and smear with a circular motion to facilitate rapid drying.

5. Stain with the safranin solution for about fifteen seconds.

6. Wash with distilled water; drain.

7. Blot dry, and examine under the oil-immersion objective.

Results:

Bacterial cells are stained pink; fatty material is bluish grey or blue-black.

Note: With reasonable care, unmounted smears will remain unchanged for at least six months.

From *J. Bact.*, 43, 717–24, 1942, Burdon, K. L., Stokes, J. C., and Kimbrough, C. E. "Studies of the common aerobic spore-forming bacilli".

SUDAN 3

A stain for blood in the spinal fluid, differentiating fresh haemorrhage from old

Solution required:

Sudan 3, saturated in 70% alcohol.

Technique:

1. Smears of spinal fluid are made on scrupulously clean slides and allowed to dry thoroughly in the air for about half an hour.

2. Without fixing, immerse in the sudan 3 solution for five to fifteen minutes, in a stoppered staining jar.

3. Rinse well with distilled water; then immerse for one minute each in four or five changes of distilled water.

4. Drain off excess water; blot and dry.

5. Mount in glycerine and examine under the oil-immersion objective.

Results:

Erythrocytes from haemorrhage more than twenty-four hours old appear with ring-like peripheries and unstained centres, while those from fresh haemorrhage in the cerebrospinal fluid are stained yellow.

SUDAN 3

For staining fat in faeces

Solution required:

Sudan 3 saturated in equal volumes of 70% alcohol and ether.

Technique:

1. A loopful of faeces is mixed with a drop of 50% alcohol.

2. Add one or two drops sudan 3 solution; then apply a coverglass before examining.

Results:

Neutral fat appears as highly refractile droplets or yellowish flakes which stain orange to orange-red.

Note: In normal faeces there is no appreciable amount of neutral fat present.

Fatty acids appear as flakes, which stain faintly; or as fine needles which tend to collect in clusters and do not take the stain.

Soaps appear as yellowish flakes, rounded or gnarled bodies, everted like the pinna of the ear, or as coarse crystals, which do not take the stain.

On warming the preparation gently, fatty acids melt, whereas the soaps do not.

In the faeces of a healthy subject the only fat elements recognizable under the microscope are yellow calcium or colourless soaps.

TETRACHROME STAIN
(MacNeal)

For differentiation of types of leucocytes

Staining solution:

Dissolve 0·3 gm. of the dry stain in 100 ml. of pure methyl alcohol by heating to 50° C. on a water bath. Shake well, and leave for four or five days with occasional shaking; then filter.

Technique:

1. Very thin, freshly prepared blood films are dried quickly at room temperature and stained immediately one to three minutes with 1 ml. of the staining solution prepared as above; then add 2 ml. buffer solution pH 6·8 and allow to stand for about five minutes.

2. Pour off the stain and wash with buffer solution pH 6·8 until the thin portions of the stained film are pink.

3. Blot carefully and examine.

Note: If it is desired to keep the films for any length of time before staining, they should be fixed immediately while still wet, with pure methyl alcohol for one minute.

Results:

Erythrocytes are stained yellowish red; polymorphonuclear neutrophiles: dark blue nuclei, reddish mauve granules, pale pink cytoplasm. Eosinophilic leucocytes: blue nuclei, red to orange-red granules, blue cytoplasm. Basophilic leucocytes: purple or dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky-blue cytoplasm. Platelets: violet to purple granules.

THIONIN (Ehrlich)

A rapid stain for rickettsia in smears

Solutions required:

A. Thionin, saturated, aqueous.

B. Sodium hydroxide 10% aqueous.

C. Phenol 2% aqueous.

Add the sodium hydroxide solution a little at a time to the thionin solution until all the dye has been precipitated; then collect the precipitate and wash with a large volume of water until the runnings show neutral reaction. Prepare a saturated solution of the precipitated dye in the 2% phenol.

Technique:

1. Air-dried smears are fixed in absolute alcohol for five minutes.

2. Pour off any excess alcohol and allow the film to dry in the atmosphere.

3. Stain in the thionin solution (prepared as above) for one half to one minute.

4. Pour off excess; drain and wash rapidly in absolute alcohol.

5. Clear in xylol and mount in Cedronol.

Results:

Rickettsia are stained deep violet. Cytoplasm is stained a light violet, while red cells are bluish green.

VAGINAL SMEAR STAIN, M.F.4

For the rapid staining of vaginal smears, sharply contrasting cornified from non-cornified cells

Technique:

1. Fix smears, while still wet, in equal parts of ether and absolute alcohol for one minute.

2. Rinse with two changes of 80% alcohol, followed by two changes of 70% alcohol.

3. Rinse by dipping the slide seven times in rapid succession in a jar of distilled water.

4. Immerse in the staining solution for two minutes.

5. Rinse with 70%, followed by 90%, alcohol.
6. Dehydrate with absolute alcohol.
7. Clear in xylol and mount.

Results:

Cornified cells, orange to red. Non-cornified cells, blue.

VAGINAL SMEAR STAIN, PX

For vaginal smears

Technique:

1. Fix smears while still wet in a mixture consisting of equal parts absolute alcohol and ether, for two minutes.
2. Rinse in 70% alcohol.
3. Rinse in 50% alcohol.
4. Rinse in water.
5. Immerse in the staining solution for three minutes.
6. Immerse in dioxane for a few seconds.
7. Wash with absolute alcohol rapidly.
8. Rinse well in xylol and mount.

Results:

Nuclei, stained red. Cornified cells, red, pink or orange. Erythrocytes, orange. Basophile celis, blue or green.

VICTORIA BLUE 4R

For elementary bodies

Solutions required:

A. Victoria Blue 4R (Lustgarten) .. 1 gm.
Distilled water .. .. .. 90 ml.
Absolute alcohol .. .. 10 ml.

B. Potassium hydroxide 0·02% aqueous.

Technique:

1. Smears are spread on scrupulously clean slides and dried in the incubator.

2. Rinse in physiological saline solution.

3. Rinse in distilled water.

4. Fix by immersing in pure methyl alcohol for an hour, in a stoppered jar.

5. Stain overnight with a freshly prepared and filtered mixture consisting of equal parts of Solutions A and B.

6. Blot and dry and mount.

Results:

Elementary bodies of vaccinia and other viruses are stained dark blue.

Reference: Gutstein, M. (1937), *J. Path. and Bact.*, **45**, no. 1, 313–4.

VICTORIA BLUE 4R

For *Treponema pallida*

Solution required:

Victoria Blue 4R (Lustgarten)
2% aqueous.

Technique:

1. Allow smears to dry in air; then fix with methyl alcohol for ten minutes.

2. Stain for five minutes in the Victoria blue solution.

3. Pour off excess stain; rinse with distilled water.

4. Drain; dry carefully but thoroughly.

5. If it is desired to preserve the specimen, mount at once in Cristalite.

Results:

Spirochaetes are stained blue; the characteristic morphology of *Treponema pallida* clearly distinguishes the organism from other spirochaetes which may be present.

VICTORIA BLUE - KERNECHTROT - LIGHT GREEN

For elementary bodies of various viruses, notably those of canary pox, ectromelia, variola, and to a lesser degree, varicella and herpes simplex

Solutions required:

A. Kernechtrot (Herzberg) .. .. 0·1 gm.
Aluminium sulphate .. .. 5 gm.
Distilled water .. .. .. 100 ml.
Dissolve by boiling; allow to cool; then filter and add:
Acetic acid 10% aqueous .. .. 0·5 ml.

B. Light green 1% aqueous .. .. 100 ml.
Acetic acid 10% .. .. .. 0·5 ml.

C. Tartaric acid 10% aqueous.

D. Victoria blue 4R 1% aqueous .. 10 ml.
Distilled water .. .. .. 90 ml.
Acetic acid 10% aqueous .. .. 5 ml.

Technique:

1. Make smears of infected tissue on scrupulously clean slides; allow to dry in the air.

2. Fix by flaming gently.

3. Wash in distilled water for ten minutes.

4. Stain in the Kernechtrot solution for an hour.

5. Rinse for thirty seconds each in two changes of distilled water.

6. Drain and allow to dry for half an hour in the atmosphere.

7. Stain in the light green solution for two minutes.

8. Rinse for five seconds in each of two changes of distilled water.

9. Immerse in the tartaric acid solution for two minutes.

10. Drain off the excess tartaric acid solution; then wash for five to ten seconds in each of two changes of distilled water.

11. Stain with the victoria blue solution for two minutes.

12. Drain off excess stain; then wash for five seconds in each of two changes of distilled water.

13. Drain and leave to dry, protected from dust, in the air or in the incubator.

Results:

Elementary bodies, blue. Cell nuclei, pink. Cell protoplasm, green.

Reference: Herzberg, K. (1934), *Zentrlb. f. Bakt. Orig.*, **131**, 358–66, "Viktoriablau zur Farbung von filtrierbarem Virus".

WRIGHT'S STAIN

For general differentiation of blood corpuscles; for malaria parasites, trypanosomes, etc.

This stain is extensively used in America instead of Leishman stain, which is generally preferred by British workers.

Best results are obtained with very thin films, and the distilled water used should be buffered to pH 6·5–7·0.

Technique:

Fixation is unnecessary unless the films are to be kept for any length of time before staining, in which case they should be fixed for five minutes with pure methyl alcohol; then blotted and dried at room temperature.

1. Place 1 ml. of the stain on a dried blood film and leave it to act for one minute; then add 2 ml. distilled water and rock the slide gently to mix.

2. Allow this diluted stain to act for three to five minutes; then pour off and wash with distilled water until the thin portion of the films appears pink to the naked eye.

3. Pour off excess stain; blot and dry at room temperature.

Results:

Erythrocytes, yellowish red. Polymorphonuclears: dark purple nuclei, reddish violet granules, pale pink cytoplasm. Eosinophiles: blue nuclei, red to orange-red granules, blue cytoplasm. Basophiles: purple to dark blue nuclei, dark purple to black granules. Lymphocytes: dark purple nuclei, sky-blue cytoplasm. Platelets,

violet to purple granules. Malarial: parasites and Leishmania chromatin, red, cytoplasm, blue. Trypanosomes: chromatin, red.

Note: The timing of the staining either before or after dilution may be altered to suit individual requirements.

Staining effects similar to Giemsa are obtained by staining for ten minutes in Wright's stain diluted with four times its volume of distilled water buffered to pH 6·5.

WRIGHT'S STAIN

For demonstrating *Trichomonas riedmuller*

Solutions required:

A. Osmic acid 2% in 0·5% chromic acid.

B. Wright's blood stain.

C. Buffer solution pH 7·0.

Technique:

1. Fresh, wet smears of the material containing *Trichomonas riedmuller* are made on slides and exposed to osmic acid fumes for ten to thirty seconds when microscopic examination should show no motility among the organisms.

2. Cover the smear with five drops of Wright's stain and fifteen drops of the buffer solution, and mix the solutions by gently rocking the slide. Allow this diluted stain to act for two to five minutes.

3. Pour off excess stain and wash thoroughly with the buffer solution.

4. Allow the smears to dry thoroughly in the air; then examine under the oil-immersion objective.

Results:

Cytoplasm, blue. Posterior portion of the axostyle, pink. Blepharoplasts, chromatin ring around the posterior part of the axostyle, endoaxostyle granules and nucleus, dark purple. The cytostome appears as a clear area at the anterior extremity of the body of the organism. The anterior flagella and those bordering the undulating membranes are stained pink.

Adapted from *J. Parasitology*, 5, 473–4, 1938, H. M. Stewart.

APPENDIX

ATOMIC WEIGHTS

Element	*Symbol*	*Atomic Weight*	*Element*	*Symbol*	*Atomic Weight*
Aluminium	Al	26·97	Krypton	Kr	83·7
Antimony	Sb	121·76	Lanthanum	La	138·92
Argon	A	39·94	Lead	Pb	207·21
Arsenic	As	74·91	Lithium	Li	6·94
Barium	Ba	137·36	Lutecium	Lu	175·0
Beryllium	Be	9·02	Magnesium	Mg	24·32
Bismuth	Bi	209·0	Manganese	Mn	54·93
Boron	B	10·82	Mercury	Hg	200·61
Bromine	Br	79·91	Molybdenum	Mo	96·0
Cadmium	Cd	112·41	Neodymium	Nd	144·27
Calcium	Ca	40·08	Neon	Ne	20·18
Carbon	C	12·01	Nickel	Ni	58·69
Cerium	Ce	140·13	Niobium	Nb	92·91
Cesium	Cs	132·91	Nitrogen	N	14·0
Chlorine	Cl	35·45	Osmium	Os	191·5
Chromium	Cr	52·01	Oxygen	O	16·0
Cobalt	Co	58·94	Palladium	Pd	106·7
Copper	Cu	63·57	Phosphorus	P	31·02
Dysprosium	Dy	162·46	Platinum	Pt	195·23
Erbium	Er	167·64	Potassium	K	39·09
Europium	Eu	152·0	Praseodymium	Pr	140·92
Fluorine	F	19·0	Protactinium	Pa	231·0
Gadolinium	Gd	156·9	Radium	Ra	226·05
Gallium	Ga	69·72	Radon	Rn	222·0
Germanium	Ge	72·6	Rhenium	Re	186·31
Gold	Au	197·2	Rhodium	Rh	102·91
Hafnium	Hf	178·6	Rubidium	Rb	85·48
Helium	He	4·0	Ruthenium	Ru	101·7
Holmium	Ho	163·5	Samarium	Sm	150·43
Hydrogen	H	1·01	Scandium	Sc	45·1
Indium	In	114·76	Selenium	Se	78·96
Iodine	I	126·92	Silicon	Si	28·06
Iridium	Ir	193·1	Silver	Ag	107·88
Iron	Fe	55·84	Sodium	Na	22·99

Strontium	..	Sr	87·63	Titanium	..	Ti	47·9
Sulphur ..	..	S	32·06	Tungsten	..	W	184·0
Tantalum	..	Ta	180·88	Uranium	..	U	238·07
Tellurium	..	Te	127·61	Vanadium	..	V	50·95
Terbium ..	..	Tb	159·2	Xenon ..	..	Xe	131·3
Thallium ..	..	Tl	204·39	Ytterbium	..	Yb	73·04
Thorium ..	..	Th	232·12	Yttrium ..	..	Y	88·92
Thulium ..	..	Tm	169·4	Zinc ..	..	Zn	65·38
Tin ..	..	Sn	118·7	Zirconium	..	Zr	91·22

CONVERSION TABLES

1 milligramme = 0·001 gramme = 0·015432 grains.
1 grain = 0·0648 grammes = 64·8 milligrammes.
1 gramme = 15·432 grains.
1 ounce (avoirdupois) = 28·3495 grammes.
1 ounce (troy) = 31·1035 grammes.
1 pound (avoirdupois) = 453·5924 grammes.
11 pounds = 5 kilogrammes.
1 ml. = 1 cc. = 16·23 minims.
10 minims = 0·616 ml.
1 fluid drachm = 3·697 ml.
1 fluid ounce = 29·673 ml.
8 fluid ounces = 236·583 ml.
1 pint (English Imperial) = 567·94 ml.
1 litre = 1,000 ml. = 33·81 fluid ounces.
1 gallon (English Imperial) = 4·5435 litres.
1 inch = 2·54 centimetres = 25·4 millimetres.
1 foot = 30·48 centimetres = 0·3048 metres.
1 yard = 0·9144 metres.
1 metre = 1·09361 yards = 39 inches (approximately).

To convert Centigrade degrees to Fahrenheit, multiply by $\frac{9}{5}$ then add 32.

To convert Fahrenheit to Centigrade degrees, deduct 32; then multiply by $\frac{5}{9}$.

FORMULAE

FIXATIVES

ALTMANN'S FLUID

Osmic acid 2%	1 volume
Potass. dichromate 5%	1 volume

BENDA'S FLUID

Osmic acid 2%	20 ml.
Chromic acid 1%	75 ml.
Glacial acetic acid	1 ml.

Note: The solution should be freshly prepared.

CHROMIC ACID

Chromium trioxide	10 gm.
Distilled water	100 ml.

GILSON'S FLUID

Mercuric chloride	5 gm.
Glacial acetic acid	1 ml.
Nitric acid, conc.	3·75 ml.
Alcohol 95%	15 ml.
Distilled water	230 ml.

KAHLE'S FLUID (DIETRICH'S FLUID)

Alcohol 95%	30 ml.
Formalin	12 ml.
Glacial acetic acid	2 ml.
Distilled water	60 ml.

MÜLLER'S FLUID

Potass. dichromate	2·5 gm.
Sodium sulphate	1 gm.
Distilled water	100 ml.

OHLMACHER'S FLUID

Absolute alcohol	64 ml.
Chloroform	12 ml.
Acetic acid glacial	4 ml.

Immediately before use add mercuric chloride, 16 gm.

PICRIC NITRIC ACID

Picric acid, aqueous, sat.	95 ml.
Nitric acid, conc.	5 ml.

PICRO-SUBLIMATE-ACETIC ACID (RATH'S FLUID)

Picric acid, saturated, aqueous	50 ml.
Mercuric chloride, hot saturated aqueous	50 ml.
Glacial acetic acid	40 ml.

PICRO-SUBLIMATE (RAHL'S FLUID)

Picric acid, saturated aqueous	50 ml.
Mercuric chloride, saturated aqueous ..	50 ml.
Distilled water	100 ml.

ZENKER-FORMOL

See Helly's fluid.

STAINS AND REAGENTS

ACETO CARMINE (SCHNEIDER)

Carmine	0·4 gm.
Distilled water	55 ml.
Heat to boiling, then add:	
Glacial acetic acid	45 ml.

Raise to boiling point again; then cool and filter.

ACID FUCHSIN (ALTMANN)

Aniline water	100 ml.
Acid fuchsin	20 gm.

ACID FUCHSIN (MALLORY)

Acid Fuchsin	0·5 gm.
Distilled water	100 ml.

ALBERT'S STAIN (for Diphtheria)

A.

Toluidine blue 0·3% aqueous	75 ml.
Methyl green 0·4% aqueous	75 ml.
Glacial acetic acid	1·5 ml.
Absolute alcohol	4 ml.

Shake well, heat to about 80° C. then allow to stand for twenty-four hours before filtering.

B.

Iodine, resublimed	1 gm.
Potassium Iodide	1·5 gm.
Distilled water	150 ml.

Dissolve the potassium Iodide in about 3 to 5 ml. of the water; then add the iodine and shake until dissolved before adding the remainder of the water.

ALUM CARMINE (MAYER)

Distilled water	50 ml.
Alum carmine powder	3·5 gm.

Shake well; then boil for a minute; cool and filter.

AMMONIA CARMINE

Ammonia carmine powder (Michrome Brand)	0·5 gm.
Distilled water	100 ml.

ANILINE BLUE, ACETIC (MASSON)

Aniline blue 1% aqueous	100 ml.
Glacial acetic acid	2 ml.

ANILINE BLUE, ALCOHOLIC

Aniline blue, alcohol soluble	1 gm.
Absolute alcohol	70 ml.
Distilled water	30 ml.

ANILINE BLUE, AQUEOUS

Aniline blue, water soluble	1 gm.
Distilled water	100 ml.

Store in a well-closed bottle to which a few drops of Chloroform or a crystal of thymol is added to inhibit the growth of air-borne organisms and consequent decomposition of the stain.

ANILINE CHLORIDE (ANILINE HYDROCHLORIDE), ACIDIFIED

Aniline chloride	5 gm.
Sulphuric acid 25%	100 ml.

ANILINE CHLORIDE (ANILINE HYDROCHLORIDE)

Aniline Hydrochloride	10 gm.
Distilled water	100 ml.

ANILINE - BLUE - ORANGE G (MALLORY)

Aniline blue-Orange G (Michrome Brand)	2·5 gm.
Distilled water	55 ml.

Dissolve, and filter.

ANILINE GENTIAN VIOLET (STIRLING)

Gentian violet	5 gm.
Aniline oil	2 ml.
Water	88 ml.
Alcohol 95%	10 ml.

Dissolve by shaking thoroughly; allow to stand twenty-four hours then filter.

ANILINE SULPHATE

Aniline Sulphate	5 gm.
Distilled water	100 ml.

ANILINE WATER

Aniline oil	5 ml.
Distilled water	100 ml.

Shake vigorously: then allow to stand. Filter immediately before use.

ANILINE XYLOL

Aniline oil	1 volume
Xylol	1 volume

BEST'S CARMINE (STOCK SOLUTION)

Potassium carbonate	1 gm.
Potassium chloride	5 gm.
Carmine	2 gm.
Distilled water	60 ml.

Heat gently for five to ten minutes until dissolved: then cool and add:

Ammonia solution (sp. gr. 0·880)	20 ml.

or:

Best's Carmine powder (Michrome Brand)	10 gm.
Distilled water	100 ml.

Heat to boiling point; cool and add:

Strong ammonia solution (sp. gr. 0·880)..	20 ml.

BORAX CARMINE (GRENACHER)

Borax	4 gm.
Carmine	3 gm.
Distilled water	100 ml.
Alcohol 70%	100 ml.

Shake the borax and carmine with the water: then boil gently for thirty minutes; allow to cool; make up to the original volume with distilled water; then add the 100 ml. 70% alcohol. Mix well and allow to stand several days before filtering.

BORAX CARMINE (MAYER)

Borax	1 gm.
Carmine	1 gm.
Distilled water	50 ml.
Absolute alcohol	50 ml.

Shake the borax and carmine with the water: then boil gently for thirty minutes; cool; make the volume up to 50 ml. with distilled water; add the alcohol; then filter.

BORREL'S BLUE

Borrel's blue powder (Michrome Brand) ..	2 gm.
Distilled water	50 ml.

Boil for five minutes: then cool and filter and make the volume up to 50 ml. with distilled water.

CARBOL FUCHSIN (ZIEHL NEELSEN)

Basic fuchsin 10% in absolute alcohol ..	10 ml.
Phenol 5% aqueous	100 ml.

Mix well and allow to stand overnight before filtering.

CARBOL GENTIAN VIOLET

Gentian violet 10% in absolute alcohol ..	10 ml.
Phenol 2% aqueous	100 ml.
Alcohol 95%	20 ml.

Mix well; then filter.

CARBOL METHYLENE BLUE

Phenol 5% aqueous	100 ml.
Methylene blue	1 gm.
Absolute alcohol	10 gm.

CARBOL THIONIN

Thionin (Ehrlich)	0·125 gm.
Phenol crystals	1 gm.
Alcohol 10%	100 ml.

Heat to dissolve, or triturate in a mortar; filter when dissolved. The solution deteriorates after a few weeks.

CARBOL TOLUIDINE BLUE

Carbol Toluidine blue powder (Michrome Brand)	2·5 gm.
Distilled water	50 ml.

CARMALUM (MAYER)

Carminic acid	1 gm.
Potash alum 5%	200 ml.

Dissolve by heating. Cool and filter. Add a crystal of thymol or a few drops of chloroform, to the stock bottle, as preservative.

COTTON BLUE - LACTOPHENOL

Cotton blue	1 gm.
Lactophenol	100 ml.

COTTON BLUE - MAGENTA LACTOPHENOL

Cotton blue 1% in lactophenol	8 ml.
Magenta 1% in lactophenol	100 ml.
Lactophenol	100 ml.

DORNER'S NEGATIVE STAIN

Nigrosin	10 gm.
Distilled water	100 ml.
Formalin	9·5 ml.

FAST-GREEN FCF IN CELLOSOLVE

Cellosolve	100 ml.
Fast-green FCF	3 gm.

Heat in a flask on a water bath for about half an hour with occasional stirring; then cool and filter.

FLAGELLA STAIN MORDANT (LOEFFLER)

Tannic acid 20% aqueous	100 ml.
Ferrous sulphate, cryst.	20 gm.
Distilled water	40 ml.
Basic fuchsin 10% in absolute alcohol	10 ml.

Dissolve the ferrous sulphate by shaking vigorously with the cold distilled water (do not heat) then add the tannic acid and basic fuchsin.

GALLOCYANIN (EINARSON)

Gallocyanin	0·3 gm.
Chrome alum	10 gm.
Distilled water	200 ml.

Boil gently for five minutes; then cool; filter, and make up to the original volume with distilled water.

GIEMSA STAIN

Giemsa stain	3·8 gm.
Pure methyl alcohol	250 ml.
Pure glycerine	250 ml.

Triturate with a mortar and pestle, adding the alcohol and glycerine in small portions at a time during a period of fifteen minutes. Transfer to a stoppered flask and allow to stand for twenty-four hours with occasional shaking; then filter.

GOODPASTURE'S ACID POLYCHROME METHYLENE BLUE

Methylene Blue	0·25 gm.
Potassium carbonate 0·25% aqueous	100 ml.

Boil for half an hour, then cool under the tap and add 0·75 ml. glacial acetic acid.

Shake well then boil gently for five to ten minutes; cool and make up the volume to 100 ml. with distilled water.

GRAM'S IODINE

Iodine pure	1 gm.
Potass. iodide	2 gm.
Water	300 ml.

Dissolve the potass. iodide in about 10 ml. of the water; then add the iodine, shake till dissolved; add the remainder of the water.

or:

Gram's Iodine powder (Michrome Brand)	1 gm.
Water	88 ml.
Hydrochloric acid, conc.	0.5 ml.

HAEMALUM (HARRIS)

Haematoxylin 0·5% aqueous	100 ml.
Potash alum	5 gm.
Mercuric chloride powder..	0·25 gm.

Heat to boiling point, then allow to cool, and filter.

HAEMALUM (MAYER)

Haematein	0·1 gm.
Absolute alcohol	5 ml.
Potash alum 5% aqueous	100 ml.

HAEMATOXYLIN (ALUM), AQUEOUS

Haematoxylin 0·25% aqueous	400 ml.
Ammonia or potash alum	20 gm.
Thymol	1 gm.

Note: The solution deteriorates after two or three months.

HAEMATOXYLIN (DELAFIELD)

Haematoxylin 3·5% in absolute alcohol ..	100 ml.
Ammonia alum 6·25% aqueous	320 ml.
Glycerine	80 ml.

Mix thoroughly and allow to ripen for at least three months; then filter before use.

HAEMATOXYLIN (EHRLICH)

Haematoxylin 2% in absolute alcohol ..	100 ml.
Potash alum 2·5% aqueous	100 ml.
Glycerine	100 ml.
Glacial acetic acid	10 ml.

Mix well; allow to ripen in a 500-ml. closed bottle for three months

or:

Dissolve 1 gm. Ehrlich haematoxylin powder (Michrome brand) in a mixture of 4 ml. glacial acetic acid, 80 ml. 50% alcohol and 40 ml. glycerine; then filter. The solution requires no ripening and is ready for immediate use.

HAEMATOXYLIN (HARRIS)

Haematoxylin 10% in absolute alcohol	5 ml.
Mercuric oxide	0·25 gm.
Potash alum 10% aqueous	100 ml.
Glacial acetic acid	4 ml.

Mix the haematoxylin and alum solutions: raise to boiling point then add the mercuric oxide and when the solution turns deep purple turn off the heat: then cool and add the acetic acid.

HAEMATOXYLIN (HEIDENHAIN)

No. 1

Iron alum	4 gm.
Distilled water	100 ml.

Dissolve by shaking. Do not heat.

No. 2

Haematoxylin 10% in absolute alcohol (ripened three to six months)	5 ml.
Distilled water	95 ml.

HAEMATOXYLIN (IRON) (ANDERSON)

No. 1

Haematoxylin 10% in absolute alcohol	5 ml.
Absolute alcohol	45 ml.
Distilled water	50 ml.
Calcium hypochloride 2% aqueous	3 ml.

No. 2

Iron alum 4% aqueous	100 ml.
Sulphuric acid conc.	3 ml.

For use, mix two volumes of solution No. 1 with one volume of No. 2; this mixture keeps for a few hours only, and should therefore be prepared only when needed for immediate use.

HAEMATOXYLIN KULTSCHITZKY

Haematoxylin 10% alcoholic	10 ml.
Acetic acid 2% aqueous	100 ml.

Dissolve the haematoxylin in the alcohol then shake thoroughly with the acetic acid solution.

HAEMATOXYLIN PHOSPHOTUNGSTIC MALLORY

Haematoxylin 10% alcoholic	1 ml.
Phosphotungstic acid 10%	20 ml.
Distilled water	80 ml.

Ripen by adding 4 drops Hydrogen peroxide (20 volumes) or 5 ml. of 1% Potassium permanganate.

HAEMATOXYLIN PHOSPHOMOLYBDIC MALLORY

Haematoxylin 1% aqueous	100 ml.
Chloral hydrate	6 gm.
Phosphomolybdic acid 10%	1 ml.

HAEMATOXYLIN REGAUD

Haematoxylin 10% in absolute alcohol	10 ml.
Glycerine	10 ml.
Distilled water	80 ml.

HAEMATOXYLIN (WEIGERT)

A. Haematoxylin 10% in absolute alcohol (ripened three to six months)	10 ml.
Absolute alcohol	90 ml.
B. Ferric chloride (hydrated) 30% aqueous	4 ml.
Hydrochloric acid	1 ml.
Distilled water	95 ml.

IODINE GREEN, ACETIC

Iodine Green 1% aqueous	99 ml.
Glacial acetic acid	1 ml.

JENNER STAIN

Jenner stain powder	0·5 gm.
Pure methyl alcohol	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool; then filter.

LEISHMAN STAIN

Leishman stain powder	0·15 gm.
Pure methyl alcohol	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool; then filter.

LIGHT GREEN IN CELLOSOLVE

Cellosolve	100 ml.
Light Green	3 gm.

Heat in a flask on a water bath for about half an hour, with occasional shaking or stirring; then cool and filter.

LITHIUM CARMINE (Orth)

Lithium carbonate, saturated aqueous	100 ml.
Carmine	5 gm.

Boil for fifteen minutes then cool and filter.

or:

Lithium Carmine (Michrome Brand)	2 gm.
Distilled water	50 ml.

Heat to boiling point; allow to cool; then filter.

LUGOL'S IODINE

Iodine pure	1 gm.
Potassium iodide	2 gm.
Water	100 ml.

MAGENTA-COTTON BLUE-LACTOPHENOL

Cotton blue-lactophenol	4 ml.
Magenta 1% aqueous	2 ml.
Lactophenol	50 ml.

MAY-GRUNWALD STAIN

May-Grunwald stain powder	0·25 gm.
Pure methyl alcohol	100 ml.

Dissolve by heating in a flask, lightly plugged with cotton-wool, on a water bath. Allow to cool, then filter.

METHYL GREEN - PYRONIN (PAPPENHEIM - UNNA)

Methyl green	0·15 gm.
Pyronin (B or G)	0·25 gm.
Phenol 0·5% aqueous solution	80 ml.
Glycerine	20 ml.
Absolute alcohol	2·5 ml.

Dissolve the powder in the phenol solution; then shake vigorously with the glycerine and alcohol: warm to dissolve; then filter.

METHYL VIOLET (JENSEN)

Methyl violet 6B	0·5 gm.
Distilled water	100ml.

METHYLENE BLUE (BORAX) (MANSON)

Methylene blue	2 gm.
Borax 5% aqueous	100 ml.

Note: Methylene blue solutions must not be heated above 60° C.

METHYLENE BLUE (BORAX) (UNNA)

Methylene blue	1 gm.
Borax 1% aqueous	100 ml.

Note: Do not heat borax methylene blue solutions over 60° C.

METHYLENE BLUE (LOEFFLER)

Methylene blue	3 gm.
Absolute alcohol	30 ml.
Potass. hydroxide 0·01% aqueous	100 ml.

METHYLENE BLUE, POLYCHROME (UNNA)

Methylene blue	1 gm.
Potass. carbonate	1 gm.
Absolute alcohol	20 ml.
Water	100 ml.

Heat to 60° C. for fifteen minutes. Cool and filter.

or:

Polychrome methylene blue (Michrome)	3 gm.
Distilled water	200 gm.
Absolute alcohol	10 ml.

Dissolve by heating to 60°C and maintain at that temperature for fifteen minutes. Cool and filter; then add the alcohol and mix thoroughly.

METHYLENE BLUE SULPHURIC (GABBET)

Distilled water	75 ml.
Sulphuric acid, conc.	25 ml.
Methylene blue	2 gm.

Pour the sulphuric acid into the water carefully, in a flask, then add the methylene blue and shake or stir to dissolve.

MUCICARMINE (MAYER)

Stock solution:

Carmine	1 gm.
Aluminium chloride, anhydrous	0·5 gm.

Mix thoroughly by grinding to a homogeneous powder in a mortar; then place the mixture in a flask with 100 ml. of 50% alcohol and boil for two and a half to five minutes on a water bath, shaking the flask at intervals. Cool under the tap at once and filter when cold.

For use, dilute one volume of this solution with nine volumes distilled water;

or:

Shake 5 gm. of Mucicarmine powder (Michrome Brand) with 200 ml. of 50% Alcohol; then heat to boiling point and continue boiling for 2½ minutes. Cool; filter.

MUCIHAEMATEIN

Mucihaematein powder (Michrome)	0·3 gm.
Glycerine	40 ml.
Distilled water	60 ml.

Dissolve by warming and shaking; then cool and filter.

NEISSER'S STAIN

A.	Methylene blue	0·2 gm.
	Absolute alcohol	4 ml.
	Glacial acetic acid	10 ml.
	Distilled water	190 ml.
B.	Bismark brown	0·2 gm.
	Distilled water	100 ml.

NEUTRAL RED (JENSEN)

Neutral red	0·1 gm.
1% acetic acid	0·2 ml.
Distilled water	100 ml.

NISSL'S METHYLENE BLUE

Venetian soap	0·175 gm.
Distilled water	80 ml.

Dissolve; then add:

Methylene Blue 2% aqueous	20 ml.

Shake vigorously then store in a well-closed bottle for at least four months, before use, shaking once or twice a week during this period.

ORCEIN (UNNA - TÄNZER)

Orcein	1 gm.
70% Alcohol	100 ml.
Hydrochloric acid, conc.	1 ml.

PARACARMINE (MAYER)

Carminic acid	1 gm.
Aluminium chloride, hydrated	0·5 gm.
Calcium chloride, anhydrous	4 gm.
Alcohol 70%	100 ml.

PHLOXIN - PROPYLENE GLYCOL

For eosinophile counts

Phloxin 0·1% in distilled water	50 ml.
Propylene glycol	50 ml.

Stated to give more consistent results with no destruction of eosinophiles.

Reference: Henneman, Wexler and Westenhaver (1949), *J. Lab. and Clin. Med.*, **34**, 1017–20.

PICRO ANILINE BLUE, ALCOHOLIC

Aniline blue alcohol soluble	1 gm.
70% alcohol	50 ml.
Picric acid, 1% in 70% alcohol	50 ml.

PICRO ANILINE BLUE, AQUEOUS

Aniline blue water soluble	0·1 gm.
Picric acid saturated, aqueous	100 ml.

PICROCARMINE

Picrocarmine powder (Michrome Brand)	1 gm.
Distilled water	50 ml.

Boil gently for five minutes: allow to cool; then filter and make up the volume to 50 ml. with distilled water.

PICRO INDIGO CARMINE

Picric acid, saturated aqueous	100 ml.
Indigo carmine	0·25 gm.

RANDOLPH'S BLOOD COUNTING FLUID

Solution A

Methylene blue	0·1 gm.
Propylene glycol	100 ml.

Solution B

Eosin, bluish	0·1 gm.
Distilled water	100 ml.

Mix equal volumes of the two solutions and use as a diluting fluid in the white cell pipette. The mixture of the blood and the diluting fluid should remain in the pipette for fifteen minutes before filling the counting chamber.

Reference: Randolph, T. G. (1943) *Proc. S. Exp. Biol. Med.*, **52**, p. 20–2.

RED CELL COUNTING FLUID (O. T. George)

Sodium citrate 3% aqueous	100 ml.
Formalin (Formaldehyde 40%)	1 ml.
Eosin, bluish	0·6 gm.

This solution stains the erythrocytes and causes the cells to lay flat in a single focal plane, thereby facilitating enumeration.

Reference: (1954), *J. Lab. and Clin. Med.*, **40**, 479–83.

SCARLET R, ALKALINE (HERZHEIMER)

Absolute alcohol	35 ml.
Distilled water	5 ml.
Sodium hydroxide 10% aqueous	10 ml.
Scarlet R	1 gm.

Place in a flask and plug the neck lightly with cotton wool. Heat on a hot water bath until a considerable portion of the dye has gone into solution; then allow to cool before filtering.

SCHORR'S STAIN

A. Harris haematoxylin.

B. Biebrich scarlet	1 gm.
Orange G	0·4 gm.
Distilled water	99 ml.
Glacial acetic acid	1 ml.

Heat the water: add the stains; stir untill dissolved allow to cool; then add acetic acid; shake well and filter.

C. phosphotungstic acid 10%	25 ml.
phosphomolybdic acid 10%	25 ml.
Distilled water	100 ml.

D. Fast green FCF aqueous 5%	5 ml.
acetic acid 1%	32 ml.
Distilled water	64 ml.

SCHORR'S STAIN
(Single solution)

Biebrich scarlet, aqueous	0·5 gm.
Orange G	0·25 gm.
Fast green FCF	0·075 gm.
Phosphotungstic acid	0·5 gm.
Phosphomolybdic acid	0·5 gm.
Glacial acetic acid	1 ml.
Alcohol 50%	100 ml.

Dissolve by warming; then allow to cool and filter.

or:

Schorr's Stain powder (Michrome)	1 gm.
Alcohol 50%	55 ml.
Glacial acetic acid	0·5 ml.

Dissolve the stain by warming with the alcohol; allow to cool; then add the acetic acid, shake well and filter.

TOLUIDINE BLUE (PONDER'S STAIN) FOR DIPHTHERIA

Toluidine blue 0·02% aqueous	100 ml.
Glacial acetic acid	1 ml.
Alcohol	5 ml.

TOLUIDINE BLUE (PUGH)

Toluidine blue	0·2 gm.
Glacial acetic acid	5 ml.
Absolute alcohol	2 ml.
Distilled water	43 ml.

REFRACTIVE INDICES

Absolute alcohol (ethyl)	1·361
Acetic acid, glacial	1·371
Acetone	1·359
Aniline oil	1·586
Aniseed oil	1·557
Aquamount	1·435
Balsam tolu	1·640
Benzene (benzol)	1·501
Benzyl benzoate	1·568

Refractive Indices (cont.)

Bergamot oil	1·464
Butyl alcohol (normal)	1·399
Cajeput oil	1·457
Canada balsam, natural, dried	1·535
Canada balsam, natural	1·521
Canada balsam, synthetic, in xylol	1·515
Canada balsam, neutral, in xylol	1·520
Carbon bisulphide	1·630
Carbon tetrachloride	1·460
Castor oil	1·490
Cedarwood oil, natural	1·510
Cedarwood oil for immersion lenses	1·506–1·518
Cedronol	1·520
Cellosolve	1·405
Chloroform	1·447
Cinnamon oil	1·567
Clove oil	1·533
Colophonium, dried	1·545
Colophonium turpentine	1·510
Creosote, beechwood	1·538
Cristalite	1·515
Crown glass	1·518
Dammar, dried	1·520
Dammar xylol	1·520
Dimethylaniline	1·558
Dimethylphthalate	1·480
Dioxane	1·423
Ethyl acetate	1·372
Ethylene glycol	1·432
Eucalyptol	1·450
Farrant's medium	1·420
Glycerine	1·460
Gum chloral (Defaur)	1·415
Glycerine albumen	1·405
Gum chloral (Berlese)	1·440
Isobutyl alcohol	1·397
Lactophenol	1·460
Lemon oil	1·527
Methyl alcohol (absolute)	1·328
Methyl benzoate	1·515

Methyl salicylate	1·537
Monobromnaphthalene-alpha	1·660
Olive oil	1·473
Optoil	1·515
Origanum oil	1·458
Paraffin, liquid	1·471
Pyridine	1·509
S.Q.D. balsam	1·515
Turpentine oil	1·473
Water (distilled)	1·333
Xylol	1·492

SATURATED SOLUTIONS OF REAGENTS

To prepare 100 ml. dissolve:

	grammes in mls. water
Ammonia alum	13 ,, 92
Aluminium chloride, hydrated	75 ,, 60
Potash alum	13·3 ,, 92
Ammon. molybdate	39 ,, 88
Ammon. oxalate	5·5 ,, 97
Aniline chloride	54 ,, 56
Aniline sulphate	6 ,, 96
Barium chloride	33 ,, 91·5
Borax	5·9 ,, 98
Boric acid	5·1 ,, 97
Chloral hydrate	120 ,, 31
Chrome alum	22 ,, 90
Chromic acid	103·5 ,, 62·5
Citric acid	87 ,, 44·5
Cobalt nitrate	125 ,, 31·5
Ferric ammon. sulphate	62 ,, 64
Ferric chloride	125 ,, 29
Ferrous ammon. sulphate	31·5 ,, 85
Ferrous sulphate	51 ,, 73
Hydroquinone	7 ,, 95
Lactose	17 ,, 90
Lithium chloride	66 ,, 62
,, citrate	47 ,, 74
,, sulphate	33 ,, 88·5
Magnesium chloride	79 ,, 47·5

	grammes in	*mls. water*
Magnesium sulphate	72 ,,	58·5
Mercuric chloride	7·5 ,,	99
Mercurous nitrate	7·7 ,,	99
Oxalic acid	13 ,,	91
Phosphomolybdic acid	135 ,,	46
Phosphotungstic acid	160 ,,	64
Potass. acetate	94 ,,	44·5
,, bromate	7·7 ,,	98
,, bromide	55 ,,	82
,, carbonate	78 ,,	74·5
,, chlorate	6 ,,	98
,, chloride	31 ,,	87
,, dichromate	14·5 ,,	95
,, ferricyanide	37 ,,	82·5
,, ferrocyanide	30·5 ,,	86
,, hydroxide	78 ,,	68
,, iodide	99 ,,	70
,, nitrate	35 ,,	85
,, oxalate	34 ,,	86
,, permanganate	7 ,,	97
,, sulphate	11·5 ,,	97
Resorcinol	68·5 ,,	45
Rochelle salts	68·5 ,,	63
Silver nitrate	164 ,,	65·5
Sodium acetate	65 ,,	53
,, bicarbonate	8·5 ,,	98
,, bisulphate	87 ,,	60
,, carbonate, anhydrous	27·5 ,,	96
,, chlorate	72 ,,	73
,, chloride	31·5 ,,	89
,, hydroxide	77 ,,	74
,, nitrate	65·5 ,,	73
,, nitrite	56·5 ,,	76
,, salicylate	65 ,,	60
,, sulphate	45·5 ,,	72·5
,, sulphite, anhydrous	28·5 ,,	95·5
,, thiosulphate	93 ,,	46
,, tungstate	73 ,,	82
Tartaric acid	76·5 ,,	54
Trichloracetic acid	148 ,,	10·7
Uranium nitrate	120 ,,	54·5
Zinc chloride	128 ,,	61

SPECIFIC GRAVITIES (at 4° C.)

Acetic acid glacial	1·049	Ethyl acetate	0·898
Acetone	0·788	Ethyl alcohol (abs. alc.)	0·816
Aniline oil	1·022	Eucalyptol	0·921
Benzene (benzol)	0·876	Isobutyl alcohol	0·806
Benzyl alcohol	1·050	Mercury	13·500
Benzyl benzoate	1·114	Methyl alcohol	0·790
Carbon bisulphide	1·260	Methyl salicylate	1·184
Carbon tetrachloride	1·589	Octyl alcohol	0·834
Chloroform	1·475	Pyridine	0·988
Dimethylaniline	0·956	Toluol	0·867
Dioxane	1·030	Xylol	0·860
Ether	0·752		

STAIN SOLUBILITIES AND MOLECULAR WEIGHTS

Notes:

The letters A, B and N indicate acid, basic and neutral dyes.

Many stains in aqueous solutions, particularly dilute aqueous solutions, are attacked and decomposed by airborne micro-organisms when kept in stock for any length of time, e.g. eosin, aniline blue. In such cases it is best to add a small crystal of thymol or a few drops of chloroform as preservative. In the case of alcoholic solutions such precautions are unnecessary, since the alcohol itself acts as a preservative, preventing the growth of such airborne micro-organisms.

Solutions of auramine decompose when heated above 35–40° C. Aqueous solutions of eosin should be prepared by using water of a slightly alkaline reaction as traces of acid cause precipitation of the acid radicle of the dye and consequent loss of staining affinity and colour. Water buffered pH 7·2–8·0 gives good results with eosin. Alkaline solutions of methylene blue decompose when heated above 60–65° C.

			Approximate percentage solubilities at 15° C.	
	Stain	*Mol. Wt.*	*In water*	*In alcohol*
A	Acid green	792·83	20·0	4·0
A	Acetyl yellow	401·22	40·00	0·01
A	Acid brown, R	400·00	1·00	0·01
A	Acid yellow, S	380·24	2·00	nil
B	Acridine orange	556·02	0·50	0·50

	Stain	Mol. Wt.	Approximate percentage solubilities at 15° C. In water	In alcohol
B ..	Acridine yellow (H 107) ..	273·31	0·50	0·75
A ..	Acid violet	483·38	3·0	nil
A ..	Alizarin	240·20	0·1	1·0
A ..	Alizarin cyanin	288·21	1·00	0·50
A ..	Alizarin red S	342·25	6·5	0·1
A ..	Alizarin saphirol	474·00	0·70	nil
A ..	Alkali blue 5B	573·61	5·0	nil
A ..	Alkali blue 6B	635·58	5·5	1·0
B ..	Amethyst violet	435·90	7·00	15·00
A ..	Amidonaphthol red, G ..	519·00	12·00	nil
A ..	Aniline blue, aqueous ..	737·71	50·0	nil
B ..	Aniline blue, alcoholic ..	490·02	nil	1·5
B ..	Auramine O	303·82	1·0	4·0
A ..	Azo blue	616·57	2·50	0·02
A ..	Azo bordeaux	286·32	5·00	0·20
A ..	Azocarmine G	433·92	1·0	0·1
A ..	Azocarmine B	681·61	2·0	0·05
A ..	Azoeosin	380·33	0·5	0·05
A ..	Azophloxin	509·42	12·0	0·02
A ..	Benzoazurin	758·00	1·50	nil
A ..	Benzopurpurin 4B ..	724·71	nil	0·20
A ..	Biebrich scarlet, aqueous ..	556·47	5·0	0·20
A ..	Biebrich scarlet, alcoholic ..	380·43	nil	0·5
B ..	Bismark brown	461·39	1·5	3·0
B ..	Bleu de nuit	490·02	nil	1·5
A ..	Bordeaux B or R	502·42	5·00	0·20
B ..	Brilliant green	482·61	5·0	8·0
A ..	Carmoisin	502·42	7·00	0·02
B ..	Celestin blue	363·79	2·0	1·5
A ..	China blue	737·71	50·0	nil
B ..	China green	364·90	10·0	8·5
A ..	China yellow	477·00	5·00	0·02
B ..	Chrysoidin 3R	262·73	0·1	1·5
B ..	Chrysoidin Y	248·71	5·5	4·8
A ..	Chlorazol azurine	758·67	1·5	0·1
A ..	Chlorazol black	781·72	6·0	0·1
A ..	Chlorophosphine	660·00	2·00	nil
A ..	Chromotrope 2R	468·37	19·0	0·15
A ..	Coeruline	554·12	1·00	0·02

	Stain	Mol. Wt.	Approximate percentage solubilities at 15° C. In water	In alcohol
A ..	Congo corinth	697·64	0·5	0·1
A ..	Congo red	696·65	5·5	0·75
A ..	Corollin, alcoholic	290·11	nil	1·25
A ..	Corollin, aqueous	335·28	8·0	4·5
A ..	Cotton blue	737·71	40·0	nil
B ..	Cresyl fast violet B ..	339·81	0·35	1·2
B ..	Cresyl fast violet BS ..	483·99	2·8	0·55
A ..	Crocein scarlet	556·47	1·50	0·50
B ..	Crystal violet	407·97	9·0	8·75
A ..	Cyanosin	829·70	10·0	4·75
B ..	Dahlia	421·99	6·0	7·5
A ..	Dianil blue 2R	820·73	5·00	0·01
A ..	Durazol yellow	660·00	2·00	nil
A ..	Eosin, yellowish	691·90	44·0	2·0
A ..	Eosin, bluish	628·09	12·0	5·0
A ..	Eosin, ethyl	697·96	nil	1·0
A ..	Eosin, scarlet	624·09	10·0	3·0
A ..	Erie garnet	697·64	0·5	0·1
A ..	Erio green	690·00	4·50	4·50
A ..	Eriochrome violet BA ..	366·00	6·00	nil
A ..	Erythrosin	628·09	10·0	5·0
B ..	Ethyl violet..	492·12	6·0	5·0
A ..	Fast acid violet	761·00	5·00	0·75
A ..	Fast green FCF	808·83	3·0	0·4
A ..	Fast light yellow	380·20	nil	1·00
A ..	Fast yellow extra	401·22	38·00	0·02
A ..	Fluorescein	376·27	40·0	4·0
A ..	Fuchsin Acid	585·54	45·00	3·00
B ..	Fuchsin Basic	361·40	1·00	8·00
A ..	Gallamine blue (bisulphite)	404·82	2·50	0·03
B ..	Gallamine blue (hydrochloride)	335·82	0·50	0·50
B ..	Gallocyanin (hydrochloride)	335·71	0·5	1·25
B ..	Gallocyanin (bisulphite) ..	380·31	3·0	0·5
B ..	Gentian blue	490·02	nil	1·5
B ..	Gentian violet	407·93	5·0	10·0
B ..	Gossypimine	322·78	4·5	3·5
A ..	Guinea green, B	735·00	5·00	5·00
A ..	Hofmann's blue	737·71	50·0	nil

	Stain	Mol. Wt.	Approximate percentage solubilities at 15° C. In water	In alcohol
B ..	Hofmann's violet	421·99	6·0	7·5
A ..	Hydrazine yellow	380·20	5·00	0·02
A ..	Indigo carmine	466·35	1·3	nil
B ..	Iodine green	364·90	8·5	8·0
B ..	Indulin alcoholic	566·09	nil	1·50
A ..	Indulin aqueous	691·54	2·5	nil
A ..	Isamine blue	735·79	5·50	1·50
B ..	Janus black	441·90	1·0	1·5
B ..	Janus green	511·05	5·0	1·0
B ..	Janus red	453·91	1·0	2·0
A ..	Light green	792·83	20·0	4·0
A ..	Lignin pink	519·00	12·00	nil
A ..	Magdala red	829·70	10·5	4·75
B .	Malachite green	364·90	10·0	8·5
A ..	Manchester yellow ..	507·30	0·20	nil
A ..	Martius yellow	507·30	0·20	nil
B ..	Meldola blue	310·00	2·00	0·01
A ..	Metachrome yellow 2RD ..	558·47	0·50	0·3
A ..	Metanil yellow	375·37	5·0	1·5
A ..	Methyl blue	799·78	50·0	nil
B ..	Methyl green OO	614·77	8·00	3·00
B ..	Methyl violet 5B	407·93	5·0	9·5
B ..	Methyl violet 6B	484·06	5·0	5·75
B ..	Methyl violet 10B	407·97	9·0	8·75
B ..	Methylene blue	319·50	9·50	6·00
B ..	Methylene green	364·84	1·5	0·1
A ..	Nacarat	502·00	7·00	0·02
A ..	Naphthalene brown ..	400·00	1·20	0·02
A ..	Naphthalene green ..	690·00	4·00	5·00
B ..	Naphthol blue R	310·00	2·00	0·02
A ..	Naphthol green, B	604·26	10·0	nil
B ..	Neutral red	288·77	4·0	1·8
B ..	New blue R	310·00	2·00	0·02
B ..	Night blue	576·00	2·25	2·25
B ..	Nile blue A (sulphate) ..	732·82	6·0	5·0
A ..	Orange G	452·37	8·0	0·22
A ..	Orange 1	350·32	1·5	0·55
A ..	Orange 2	350·32	3·0	0·15
A ..	Orange 3	327·33	0·5	1·0

	Stain	Mol. Wt.	Approximate percentage solubilities at 15° C. In water	In alcohol
A ..	Orange 4	375·37	0·15	0·2
A ..	Orseillin BB	584·54	1·50	2·00
A ..	Oxydianil yellow	660·00	2·00	nil
A ..	Patent blue, A	1347·61	4·0	7·5
A ..	Patent blue, V	1159·38	5·0	3·0
B ..	Phenosafranin	322·78	4·0	3·75
B ..	Phenylene blue	310·00	2·00	0·01
A ..	Phloxine	829·70	10·5	5·0
A ..	Picric acid	229·10	1·0	8·0
A ..	Polar yellow 5G	609·00	0·50	0·30
A ..	Ponceau 2R	480·42	5·0	0·1
A ..	Ponceau S	556·47	5·0	0·5
A ..	Ponceau de xylidine ..	480·42	5·0	0·1
A ..	Ponceau de xylidine fat stain	276·32	nil	0·4
A ..	Pontacyl carmine 2B ..	519·00	12·00	nil
A ..	Pontacyl carmine 6B ..	519·00	12·00	nil
A ..	Pontacyl green	690·00	5·00	5·00
A ..	Pontacyl violet	761·00	1·00	5·00
A ..	Pontamine sky blue 5BX ..	992·80	8·0	0·02
A ..	Purpurin	256·20	nil	0·7
B ..	Pyronin, B	358·90	10·0	0·5
B ..	Pyronin G, ...	302·86	9·00	0·60
A ..	Quinalizarin	272·20	nil	0·5
A ..	Quinizarin blue	431·00	0·80	nil
A ..	Quinoline yellow	477·00	5·00	0·01
A ..	Resorcin yellow	316·21	1·50	nil
B ..	Rhodamine B	479·00	2·0	1·75
B ..	Rhodamine 6G	450·94	1·5	6·5
B ..	Rhoduline orange	556·02	0·50	0·50
B ..	Rhoduline violet	364·87	3·00	20·00
A ..	Rose bengale	1017·71	30·0	7·0
A ..	Rosolic acid	290·11	nil	1·25
A ..	Rosolic acid, sodium salt ..	335·28	8·0	4·5
A ..	Rubin S	585·54	45·00	3·00
B ..	Safranin, O..	332·78	4·5	3·5
A ..	Scarlet R	380·43	nil	0·5
B ..	Setoglaucine	399·35	5·50	5·50
A ..	Solochrome black T ..	461·32	1·50	0·02
A ..	Solochrome blue	474·00	0·70	nil

	Stain	*Mol. Wt.*	*Approximate percentage solubilities at 15° C.* *In water*	*In alcohol*
A ..	Solochrome violet R ..	566·00	6·00	nil
A ..	Soluble blue	737·71	40·0	nil
A ..	Solway purple	431·00	0·80	nil
N ..	Sudan black	456·17	nil	0·25
A ..	Sudan brown	298·32	nil	2·0
A ..	Sudan 1	248·26	nil	0·25
A ..	Sudan 2	252·30	nil	0·30
A ..	Sudan 3	352·38	nil	0·15
A ..	Sudan 4	352·59	nil	0·5
A ..	Tartrazine	511·37	6·0	nil
B ..	Thioflavine, T	310·85	2·0	1·0
B ..	Thionin (Ehrlich)	263·74	1·0	1·0
A ..	Titan yellow	695·70	1·5	nil
B ..	Toluidine blue	305·81	3·5	1·5
B ..	Toluylene blue	290·79	8·0	2·5
A ..	Tropoeolin O	316·21	1·50	nil
A ..	Tropoeolin, OO	375·19	0·15	0·2
A ..	Tropoeolin, OOO No. 1 ..	350·60	2·4	0·55
A ..	Tropoeolin, OOO No. 2 ..	350·32	3·0	0·15
A ..	Trypan blue	960·80	1·0	0·02
B ..	Turquoise blue	423·93	7·00	15·00
A ..	Uranin	376·27	40·0	4·0
B ..	Victoria blue B	506·06	4·5	8·5
B ..	Victoria blue 4R	520·09	3·0	20·0
B ..	Victoria blue R	458·02	0·5	4·0
A ..	Violamine 3B	731·50	5·00	10·00
A ..	Vital new red	782·75	1·3	nil
A ..	Water blue	799·78	40·0	nil
A ..	Wool green, S	576·60	4·0	nil
A ..	Wool violet 4BN	761·00	1·00	5·00
A ..	Xylidine red	504·50	4·70	nil

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